

Date: August 27, 1998
Case Docket No. HS 102
Express Mail No. EM500208741US

ASSISTANT COMMISSIONER FOR PATENTS
Washington, D.C. 20231

Submitted herewith for filing is the patent application of
Inventors: Gary A. Bannon, A. Wesley Burks, Hugh Sampson, Howard Sosin
For: **METHODS AND REAGENTS FOR DECREASING CLINICAL REACTION TO ALLERGY**

Enclosed are:

- ☒ 7 sheets of informal drawings.
- ☐ An Assignment of the invention to _____
- ☐ A certified copy of a _____ application.
- ☒ An unexecuted Declaration
- ☐ A verified statement to establish small entity status under 37 CFR 1.9 and 37 CFR 1.27
- ☐ Request for Filing a Divisional Application under 37 CFR §1.60.

The filing fee has been calculated as shown below:

| | (Col. 1) | (Col. 2) |
|--|-----------|-----------|
| FOR: | NO. FILED | NO. EXTRA |
| BASIC FEE | | |
| TOTAL CLAIMS | 75-20 = | 55 |
| INDEP CLAIMS | 4-3 = | 1 |
| <input checked="" type="checkbox"/> MULTIPLE DEPENDENT CLAIM PRESENTED | | |

* If the difference in Col. 1 is less than zero, enter "0" in Col. 2.

| SMALL ENTITY | | OR | OTHER THAN A SMALL ENTITY | |
|--------------|-------|----|---------------------------|--------|
| RATE | FEE | | RATE | FEE |
| | \$395 | OR | | \$790 |
| x 11 = | \$ | OR | x 22 = | \$1210 |
| x 41 = | \$ | OR | x 82 = | \$ 82 |
| x135 = | \$ | OR | x270 = | \$270 |
| TOTAL | \$ | OR | TOTAL | \$2352 |

- ☒ Please charge my Deposit Account No. 01-2507 in the amount of \$1,176.00. A duplicate copy of this sheet is enclosed.
- ☒ A check in the amount of \$1,176.00 is enclosed.
- ☒ The Commissioner is hereby authorized to charge payment of the following fees associated with this communication or credit any overpayment to Deposit Account No. 01-2507. A duplicate copy of this sheet is enclosed.
- ☒ Any additional filing fees required under 37 CFR 1.16.
- ☒ Any patent application processing fees under 37 CFR 1.17.
- ☒ The Commissioner is hereby authorized to charge payment of the following fees during the pendency of this application or credit any overpayment to Deposit Account No. 01-2507. A duplicate copy of this sheet is enclosed.
- ☒ Any patent application processing fees under 37 CFR 1.17.
- ☐ The issue fee set in 37 CFR 1.18 at or before mailing of the Notice of Allowance, pursuant to 37 CFR 1.311(b).
- ☒ Any filing fees under 37 CFR 1.16 for the presentation of extra claims.

Respectfully submitted,

Robert A. Hodges, Reg. No. 41,074

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Gary A. Bannon, A. Wesley Burks, Hugh Sampson,
Howard Sosin

Serial No: Express Mail Label No.
EM 500 208 741 US
Filed: August 27, 1998 Date of Deposit
August 27, 1998

For: **METHODS AND REAGENTS FOR DECREASING CLINICAL
REACTION TO ALLERGY**

Assistant Commissioner
for Patents
Washington, D.C. 20231

EXPRESS MAIL TRANSMITTAL LETTER
FOR PATENT APPLICATION AND CERTIFICATE OF MAILING

Sir:

Pursuant to 35 U.S.C. § 21(a) as amended by Public Law 97-247 and 37 C.F.R. § 1.10, Gary A. Bannon, A. Wesley Burks, Hugh Sampson and Howard Sosin enclose for filing the attached patent application entitled "METHODS AND REAGENTS FOR DECREASING CLINICAL REACTION TO ALLERGY". The application includes 1 page of Abstract, 29 pages of Specification, 5 pages of claims, 7 sheets of informal drawings, 19 pages of sequence listing and an unexecuted Declaration. A check in the amount of \$1,176.00 to cover the filing fee is also enclosed. A Verified Statement Claiming Small Entity Status and Assignments will be submitted shortly.

The Commissioner is hereby authorized to charge our deposit order account no. 01-2507 in the amount of \$1,176.00, which

Title: "METHODS AND REAGENTS FOR DECREASING CLINICAL REACTION TO ALLERGY"
By: Gary A. Bannon, A. Wesley Burks, Hugh Sampson, Howard Sosin
Filed: August 27, 1998
EXPRESS MAIL TRANSMITTAL FOR PATENT APPLICATION

represents the difference between the filing fee for a large entity and a small entity.

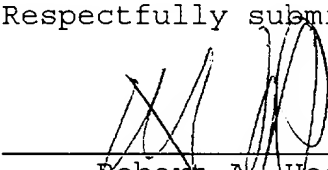
This application is being filed on by mailing the application to the Assistant Commissioner for Patents, Washington, D.C. 20231 via the United States Postal Service "Express Mail Post Office to Addressee" Service under 37 C.F.R. § 1.10. The Express Mail Label No. appears in the heading of this paper which is attached to the application papers pursuant to 37 C.F.R. § 1.10(B).

The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Account No. 01-2507. To facilitate this process, applicants have enclosed a duplicate of this letter.

All correspondence concerning this application should be mailed to:

Patrea L. Pabst, Esq.
ARNALL GOLDEN & GREGORY LLP
2800 One Atlantic Center
1201 West Peachtree Street
Atlanta, GA 30309-3450

Respectfully submitted,



Robert A. Hodges
Reg. No. 41,074

Date: August 27, 1998
ARNALL GOLDEN & GREGORY LLP
2800 One Atlantic Center
1201 West Peachtree Street
Atlanta, GA 30309-3450
(404) 873 8794

Title: "METHODS AND REAGENTS FOR DECREASING CLINICAL REACTION TO ALLERGY"
By: Gary A. Bannon, A. Wesley Burks, Hugh Sampson, Howard Sosin
Filed: August 27, 1998
EXPRESS MAIL TRANSMITTAL FOR PATENT APPLICATION

CERTIFICATE OF MAILING UNDER 37 CFR § 1.10

I hereby certify that this Express Mail Transmittal Letter for Patent Application and any documents referred to as attached therein are being deposited with the United States Postal Service on this date, August 27, 1998 in an envelope as "Express Mail Post Office to Addressee" service under 37 CFR § 1.10, Mailing Label Number EM 500 208 741 US, addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Date: August 27, 1998



Jean Hicks

APPLICATION
FOR
UNITED STATES LETTERS PATENT

BY
GARY A. BANNON

A. WESLEY BURKS

HUGH SAMPSON

AND

HOWARD SOSIN

FOR
METHODS AND REAGENTS FOR DECREASING
CLINICAL REACTION TO ALLERGY

METHODS AND REAGENTS FOR DECREASING CLINICAL REACTION TO ALLERGY

Background of the Invention

The United States government has rights in this invention by virtue of grants from the National Institute of Health RO1-AI33596.

Priority is claimed to U.S. Serial No. 60/074,590 filed February 13, 1998, entitled "Mutagenized IgE-Binding Epitopes for Ara H 1, by A. Wesley Burks, Jr., Ricki M. Helm, Gael Cockrell, Gary A. Bannon, J. Steven Stanley, and David Shin; U.S. Serial No. 60/074,624 filed February 13, 1998, entitled "Peanut Allergen Ara H 3" by A. Wesley Burks, Jr., Hugh Sampson, Gary A. Bannon, Shau-Ku Huang, and Patrick A. Rabjohn; U.S. Serial No. 60/074633 filed February 13, 1998, entitled "Mutagenized IgE-Binding Epitopes for Ara H 2" by A. Wesley Burks, Jr., Ricki M. Helm, Gael Cockrell, Gary A. Bannon, J. Steven Stanley, and Nina King; and under 35 U.S.C. §§ 120 and 371 to PCT/US96/15222 entitled "Peanut Allergens and Methods" filed September 23, 1996, by University of Arkansas.

Allergic disease is a common health problem affecting humans and companion animals (mainly dogs and cats) alike. Allergies exist to foods, molds, grasses, trees, insects, pets, fleas, ticks and other substances present in the environment. It is estimated that up to 8% of young children and 2% of adults have allergic reactions just to foods alone. Some allergic reactions (especially those to foods and insects) can be so severe as to be life threatening. Problems in animals tend to be less severe, but very common. For example, many dogs and cats have allergies to flea saliva proteins, grasses, and other common substances present in the environment.

Allergy is manifested by the release of histamines and other mediators of inflammation by mast cells which are triggered into action when IgE

antibodies bound to their receptors on the mast cell surface are cross linked by antigen. Other than avoidance, and drugs (e.g. antihistamines, decongestants, and steroids) that only treat symptoms and can have unfortunate side effects and often only provide temporary relief, the only currently medically accepted treatment for allergies is immunotherapy. Immunotherapy involves the repeated injection of allergen extracts, over a period of years, to desensitize a patient to the allergen. Unfortunately, traditional immunotherapy is time consuming, usually involving years of treatment, and often fails to achieve its goal of desensitizing the patient to the allergen. Furthermore, it is not the recommended treatment for food allergies, such as peanut allergies, due to the risk of anaphylaxis.

Noon (Noon, *Lancet* 1911; 1:1572-73) first introduced allergen injection immunotherapy in 1911, a practice based primarily on empiricism with non-standardized extracts of variable quality. More recently the introduction of standardized extracts has made it possible to increase the efficacy of immunotherapy, and double-blind placebo-controlled trials have demonstrated the efficacy of this form of therapy in allergic rhinitis, asthma and bee-sting hypersensitivity (BSAC Working Party, *Clin. Exp. Allergy* 1993; 23:1-44). However, increased risk of anaphylactic reactions has accompanied this increased efficacy. For example, initial trials of immunotherapy to food allergens has demonstrated an unacceptable safety:efficacy ratio (Oppenheimer et al. *J Allergy Clin. Immun.* 1992; 90:256-62; Sampson, *J. Allergy Clin. Immun.* 1992; 90:151-52; Nelson et al. *J. Allergy Clin. Immun.* 1996; 99:744-751). Results like these have prompted investigators to seek alternative forms of immunotherapy as well as to seek other forms of treatment.

Initial trials with allergen-non-specific anti-IgE antibodies to deplete the patient of allergen-specific IgE antibodies have shown early promise (Boulet, et al. 1997; 155:1835-1840; Fahy, et al. *American J Respir. Crit.*

Care Med. 1997; 155:1828-1834; Demoly P. and Bousquet J. *American J Resp. Crit. Care Med.* 1997; 155:1825-1827). On the other hand, trials utilizing immunogenic peptides (representing T cell epitopes) have been disappointing (Norman, et al. *J. Aller. Clin. Immunol.* 1997; 99:S127).
Another form of allergen-specific immunotherapy which utilizes injection of plasmid DNA (Raz et al. *Proc. Nat. Acad. Sci. USA* 1994; 91:9519-9523; Hz et al. *Int. Immunol.* 1996; 8:1405-1411) remains unproven.

There remains a need for a safe and efficacious therapy for allergies, especially those where traditional immunotherapy is ill advised due to risk to the patient or lack of efficacy. There is also a need for alternatives to therapies, for example, by creating foods, materials or substances that do not include the allergens that are most problematic, or which contain modified allergens which do not elicit the same reaction. While the technology to make genetically engineered plants and animals is at this point well established, useful modifications would require understanding how allergens can be modified so that they retain the essential functions for the plants' and animals' nutritional value, taste characteristics, etc., but no longer elicit as severe an allergic response.

It is therefore an object of the present invention to provide a method for decreasing the allergenicity of allergens either by modifying the allergen itself or by producing a compound that would mask the epitope and thus prevent binding of IgE.

It is a further object of the present invention to provide allergens that elicit fewer IgE mediated responses.

It is still another object of the present invention to provide a method to make genetically engineered plants and animals that elicit less of an allergic response than the naturally occurring organisms.

Summary of the Invention

It has been determined that allergens, which are characterized by both humoral (IgG and IgE) and cellular (T cell) binding sites, can be made less allergenic by modifying the IgE binding sites. The IgE binding sites can be eliminated by masking the site with a compound that would prevent IgE binding or by altering as little as a single amino acid within the protein to eliminate IgE binding. The method allows the protein to be altered as minimally as possible, (i.e. only within the IgE-binding sites) while retaining the ability of the protein to activate T cells and, optionally, to bind IgG. Binding sites are identified using known techniques, such as by binding with antibodies in pooled sera obtained from individuals known to be immunoreactive with the allergen to be modified. Proteins that are modified to alter IgE binding are screened for binding with IgG and/or activation of T cells.

Peanut allergens (Ara h 1, Ara h 2, and Ara h 3) have been used in the examples to demonstrate alteration of IgE binding sites while retaining binding to IgG and activation of T cells. The critical amino acids within each of the IgE binding epitopes of the peanut protein that are important to immunoglobulin binding were determined. Substitution of even a single amino acid within each of the epitopes led to loss of IgE binding. Although the epitopes shared no common amino acid sequence motif, the hydrophobic residues located in the center of the epitope appeared to be most critical to IgE binding.

Standard techniques such as a skin test for wheal and flare formation can be used to assess decreased allergenicity of modified proteins, created as described in the examples. The modified allergens can also be tested for binding to IgG and proliferation of T cells, and modified allergens selected for optimal stimulation of T cells and binding IgG.

The immunotherapeutics can be delivered by standard techniques,

using injection, by aerosol, sublingually, topically (including to a mucosal surface), and by gene therapy (for example, by injection of the gene encoding the immunotherapeutic into muscle or skin where it is transiently expressed for a time sufficient to induce tolerance).

This method and the criteria for identifying and altering allergens can be used to design useful proteins (including nucleotide molecules encoding the proteins) for use in immunotherapy, to make a vaccine and to genetically engineer organisms such as plants and animals which then produce proteins with less likelihood of eliciting an IgE response. Techniques for engineering plants and animals are well known. Based on the information obtained using the method described in the examples, one can engineer plants or animals to cause either site specific mutations in the gene encoding the protein(s) of interest, or to knock out the gene and then insert the gene encoding the modified protein.

Brief Description of the Drawings

Figure 1 shows an example of how IgE binding epitopes were mapped to a specific amino acid sequence on the Ara h 1 allergen.

Figure 2 shows how IgE binding epitopes were mapped to a specific amino acid sequence on the Ara h 2 allergen.

Figure 3 shows how IgE binding epitopes were mapped to a specific amino acid sequence on the Ara h 3 allergen.

Figure 4 shows how amino acids critical to IgE binding were identified.

Figure 5A shows the location of altered residues within the Ara h 2 amino acid sequence (SEQ ID NO 4).

Figure 5B shows the effect the modified Ara h 2 protein has on IgE binding.

Figure 5C shows the effect the modified Ara h 2 protein has on IgG binding.

Figure 6 shows the results of T-cell proliferation assays using the wild-type and modified Ara h 2 protein.

Detailed Description of the Invention

Definitions

The following definitions are used herein.

An antigen is a molecule that elicits production of antibody (a humoral response) or an antigen-specific reaction with T cells (a cellular response).

An allergen is a subset of antigens which elicits IgE production in addition to other isotypes of antibodies.

An allergic reaction is one that is IgE mediated with clinical symptoms primarily involving the cutaneous (urticaria, angiodema, pruritus), respiratory (wheezing, coughing, laryngeal edema, rhinorrhea, watery/itching eyes), gastrointestinal (vomiting, abdominal pain, diarrhea), and cardiovascular (if a systemic reaction occurs) systems.

An epitope is a binding site including an amino acid motif of between approximately six and fifteen amino acids which can be bound by either an immunoglobulin or recognized by a T cell receptor when presented by an antigen presenting cell in conjunction with the major histocompatibility complex (MHC). A linear epitope is one where the amino acids are recognized in the context of a simple linear sequence. A conformational epitope is one where the amino acids are recognized in the context of a particular three dimensional structure.

An immunodominant epitope is one which is bound by antibody in a large percentage of the sensitized population or where the titer of the antibody is high, relative to the percentage or titer of antibody reaction to other epitopes present in the same protein.

A decreased allergic reaction is characterized by a decrease in clinical symptoms following treatment of symptoms associated with exposure to an

allergen, which can involve respiratory, gastrointestinal, skin, eyes, ears and mucosal surfaces in general.

An antigen presenting cell (an APC) is a cell which processes and presents peptides to T cells to elicit an antigen-specific response.

Immunostimulatory sequences are oligodeoxynucleotides of bacterial, viral or invertebrate origin that are taken-up by APCs and activate them to express certain membrane receptors (e.g., B7-1 and B7-2) and secrete various cytokines (e.g., IL-1, IL-6, IL-12, TNF). These oligodeoxynucleotides containing unmethylated CpG motifs cause brisk activation and when injected into animals in conjunction with antigen, appear to skew the immune response to a Th1-type response. See, for example, Yamamoto, et al., *Microbiol. Immunol.* 36, 983 (1992); Krieg, et al., *Nature* 374, 546-548 (1995); Pisetsky, *Immunity* 5, 303 (1996); and Zimmerman, et al., *J. Immunol.* 160, 3627-3630 (1998).

I. Diagnostic and Therapeutic Reagents.

The first step in making the modified allergen is to identify IgE binding sites and/or immunodominant IgE binding sites. The second step is to mutate one or more of the IgE binding sites, preferably including at a minimum one of the immunodominant sites, or to react the allergen with a compound that selectively blocks binding to one or more of the IgE binding sites. The third step is to make sufficient amounts of the modified allergen for administration to persons or animals in need of tolerance to the allergen, where the modified allergen is administered in a dosage and for a time to induce tolerance, or for diagnostic purposes. The modified allergen can be administered by injection, or in some cases, by ingestion or inhalation.

A. Allergens.

Many allergens are known that elicit allergic responses, which may range in severity from mildly irritating to life-threatening. Food allergies are mediated through the interaction of IgE to specific proteins contained within

the food. Examples of common food allergens include proteins from peanuts, milk, grains such as wheat and barley, soybeans, eggs, fish, crustaceans, and mollusks. These account for greater than 90% of the food allergies (Taylor, Food Techn. 39, 146-152 (1992). The IgE binding epitopes from the major allergens of cow milk (Ball, et al. (1994) *Clin. Exp. Allergy*, 24, 758-764), egg (Cooke, S.K. and Sampson, H.R. (1997) *J. Immunol.*, 159, 2026-2032), codfish (Aas, K., and Elsayed, S. (1975) *Dev. Biol. Stand.* 29, 90-98), hazel nut (Elsayed, et al. (1989) *Int. Arch. Allergy Appl. Immunol.* 89, 410-415), peanut (Burks et al., (1997) *Eur. J. Biochemistry*, 245:334-339; Stanley et al., (1997) *Archives of Biochemistry and Biophysics*, 342:244-253), soybean (Herein, et al. (1990) *Int. Arch. Allergy Appl. Immunol.* 92, 193-198) and shrimp (Shanty, et al. (1993) *J. Immunol.* 151, 5354-5363) have all been elucidated, as have others. Other allergens include proteins from insects such as flea, tick, mite, fire ant, cockroach, and bee as well as molds, dust, grasses, trees, weeds, and proteins from mammals including horses, dogs, cats, etc.

The majority of allergens discussed above elicit a reaction when ingested, inhaled, or injected. Allergens can also elicit a reaction based solely on contact with the skin. Latex is a well known example. Latex products are manufactured from a milky fluid derived from the rubber tree, *Hevea brasiliensis* and other processing chemicals. A number of the proteins in latex can cause a range of allergic reactions. Many products contain latex, such as medical supplies and personal protective equipment. Three types of reactions can occur in persons sensitive to latex: irritant contact dermatitis, and immediate systemic hypersensitivity. Additionally, the proteins responsible for the allergic reactions can fasten to the powder of latex gloves. This powder can be inhaled, causing exposure through the lungs. Proteins found in latex that interact with IgE antibodies were characterized by two-dimensional electrophoresis. Protein fractions of 56, 45, 30, 20, 14, and

less than 6.5 kd were detected (Posch A. et al., (1997) *J. Allergy Clin. Immunol.* 99(3), 385-395). Acidic proteins in the 8-14 kd and 22 - 24 kd range that reacted with IgE antibodies were also identified (Posch A. et al., (1997) *J. Allergy Clin. Immunol.* 99(3), 385-395. The proteins prohevein and hevein, from hevea brasiliensis, are known to be major latex allergens and to interact with IgE (Alenius, H., et al., *Clin. Exp. Allergy* 25(7), 659-665; Chen Z., et al., (1997) *J. Allergy Clin. Immunol.* 99(3), 402-409). Most of the IgE binding domains have been shown to be in the hevein domain rather than the domain specific for prohevein (Chen Z., et al., (1997) *J. Allergy Clin. Immunol.* 99(3), 402-409). The main IgE-binding epitope of prohevein is thought to be in the N-terminal, 43 amino acid fragment (Alenius H., et al., (1996) *J. Immunol.* 156(4), 1618-1625). The hevein lectin family of proteins has been shown to have homology with potato lectin and snake venom disintegrins (platelet aggregation inhibitors) (Kielisqewski, M.L., et al., (1994) *Plant J.* 5(6), 849-861).

B. Identification of IgE Binding Sites.

Allergens typically have both IgE and IgG binding sites and are recognized by T cells. The binding sites can be determined either by using phage display libraries to identify conformational epitopes (Eichler and Houghten, (1995) *Molecular Medicine Today* 1, 174-180; Jensen-Jarolim et al., (1997) *J. Appl. Clin. Immunol.* 101, 5153a) or by using defined peptides derived from the known amino acid sequence of an allergen (see examples below), or by binding of whole protein or protein fragments to antibodies, typically antibodies obtained from a pooled patient population known to be allergic to the allergen. It is desirable to modify allergens to diminish binding to IgE while retaining their ability to activate T cells and in some embodiments by not significantly altering or decreasing IgG binding capacity. This requires modification of one or more IgE binding sites in the allergen.

A preferred modified allergen is one that can be used with a majority

of patients having a particular allergy. Use of pooled sera from allergic patients allows determination of one or more immunodominant epitopes in the allergen. Once some or all of the IgE binding sites are known, it is possible to modify the gene encoding the allergen, using site directed mutagenesis by any of a number of techniques, to produce a modified allergen as described below, and thereby express modified allergens. It is also possible to react the allergen with a compound that achieves the same result as the selective mutation, by making the IgE binding sites inaccessible, but not preventing the modified allergen from activating T cells, and, in some embodiments, by not significantly altering or decreasing IgG binding.

Assays to assess an immunologic change after the administration of the modified allergen are known to those skilled in the art. Conventional assays include RAST (Sampson and Albergo, 1984), ELISAs (Burks, et al. 1986) immunoblotting (Burks, et al. 1988), and *in vivo* skin tests (Sampson and Albergo 1984). Objective clinical symptoms can be monitored before and after the administration of the modified allergen to determine any change in the clinical symptoms.

It may be of value to identify IgEs which interact with conformational rather than linear epitopes. Due to the complexity and heterogeneity of patient serum, it may be difficult to employ a standard immobilized allergen affinity-based approach to directly isolate these IgEs in quantities sufficient to permit their characterization. These problems can be avoided by isolating some or all of the IgEs which interact with conformational epitopes from a combinatorial IgE phage display library.

Steinberger et al. (Steinberger, P., Kraft D. and Valenta R. (1996) "Construction of a combinatorial IgE library from an allergic patient: Isolation and characterization of human IgE Fabs with specificity for the major Timothy Grass pollen antigen," *Phl p. 5 J. Biol. Chem.* 271, 10967-10972) prepared a combinatorial IgE phage display library from mRNA

isolated from the peripheral blood mononuclear cells of a grass allergic patient. Allergen-specific IgEs were selected by panning filamentous phage expressing IgE Fabs on their surfaces against allergen immobilized on the wells of 96 well microtiter plates. The cDNAs were then isolated from allergen-binding phage and transformed into E coli for the production of large quantities of monoclonal, recombinant, allergen-specific IgE Fabs.

If native allergen or full length recombinant allergen is used in the panning step to isolate phage, then Fabs corresponding to IgEs specific for conformational epitopes should be included among the allergen-specific clones identified. By screening the individual recombinant IgE Fabs against denatured antigen or against the relevant linear epitopes identified for a given antigen, the subset of conformation-specific clones which do not bind to linear epitopes can be defined.

To determine whether the library screening has yielded a complete inventory of the allergen-specific IgEs present in patient serum, an immunocompetition assay can be performed. Pooled recombinant Fabs would be preincubated with immobilized allergen. After washing to remove unbound Fab, the immobilized allergen would then be incubated with patient serum. After washing to remove unbound serum proteins, an incubation with a reporter-coupled secondary antibody specific for IgE Fc domain would be performed. Detection of bound reporter would allow quantitation of the extent to which serum IgE was prevented from binding to allergen by recombinant Fab. Maximal, uncompetited serum IgE binding would be determined using allergen which had not been preincubated with Fab or had been incubated with nonsense Fab. If IgE binding persists in the face of competition from the complete set of allergen-specific IgE Fab clones, this experiment can be repeated using denatured antigen to determine whether the epitopes not represented among the cloned Fabs are linear or conformational.

Production of Recombinant or Modified Allergens

A modified allergen will typically be made using recombinant techniques. Expression in a procaryotic or eucaryotic host including bacteria, yeast, and baculovirus-insect cell systems are typically used to produce large (mg) quantities of the modified allergen. It is also possible to make the allergen synthetically, if the allergen is not too large, for example, less than about 25-40 amino acids in length.

Production of Transgenic Plants and Animals

Transgenic plants or animals expressing the modified allergens have two purposes. First, they can be used as a source of modified allergen for use in immunotherapy and second, appropriately modified plants or animals can be substituted for the original plant or animal, making immunotherapy unnecessary. Furthermore, it is possible that eating modified peanuts or cod fish, for example, could have either or both of two effects: (1) not imparting an allergic response on their own and (2) conferring protection from the unmodified source by acting as an immunotherapeutic agent for the unmodified source. Methods for engineering of plants and animals are well known and have been for a decade. For example, for plants see Day, (1996) *Crit. Rev. Food Sci. & Nut.* 36(S), 549-567, the teachings of which are incorporated herein. See also Fuchs and Astwood (1996) *Food Tech.* 83-88. Methods for making recombinant animals are also well established. See, for example, Colman, A" Production of therapeutic proteins in the milk of transgenic livestock" (1998) *Biochem. Soc. Symp.* 63, 141-147; Espanion and Niemann, (1996) *DTW Dtxch Tierarztl Wochenschr* 103(8-9), 320-328; and Colman, *Am. J. Clin. Nutr.* 63(4), 639S-645S, the teachings of which are incorporated herein. One can also induce site specific changes using homologous recombination and/or triplex forming oligomers. See, for example, Rooney and Moore, (1995) *Proc. Natl. Acad. Sci. USA* 92, 2141-2149; Agrawal, et al., *BioWorld Today*, vol. 9, no. 41, p. 1"Chimeriplasty -

Gene Surgery, Not Gene Therapy - Fixes Flawed Genomic Sequences" David N. Leff.

Production and Screening of Compounds blocking IgE Binding Sites

Once the IgE binding sites have been identified, it is also possible to block or limit binding to one or more of these sites by reacting the allergen with a compound that does not prevent the allergen from activating T cells, and in some embodiments does not significantly alter or decrease IgG binding capacity, resulting in a modified allergen similar in functionality to that produced by mutation. There are two principal ways to obtain compounds which block IgE binding sites: combinatorial libraries and combinatorial chemistry.

Identification of Compounds That Mask IgE Binding Sites through Application of Combinatorial Chemistry

In some cases it may be preferable to utilize non-peptide compounds to block binding of IgE to the allergen by masking the IgE binding epitope. This can be accomplished by using molecules that are selected from a complex mixture of random molecules in what has been referred to as "*in vitro* genetics" or combinatorial chemistry (Szostak, *TIBS* 19:89, 1992). In this approach a large pool of random and defined sequences is synthesized and then subjected to a selection and enrichment process. The selection and enrichment process involves the binding of the IgE binding epitopes to a solid support, followed by interaction with the products of various combinatorial libraries. Those molecules which do not bind these molecules at all are removed immediately by elution with a suitable solvent. Those molecules which bind to the epitopes will remain bound to the solid support, whereas, unbound compounds will be removed from the column. Those compounds bound to the column can be removed, for example, by competitive binding. Following removal of these compounds, the compounds which have bound can be identified, using methodology well known to those of skill in the art,

to isolate and characterize those compounds which bind to or interact with IgE binding epitopes. The relative binding affinities of these compounds can be compared and optimum compounds identified using competitive binding studies which are well known to those of skill in the art.

Identification of Compounds That Interact with IgE Binding Sites through Application of Combinatorial Phage Display Libraries

Recombinant, monoclonal Fabs directed against conformational epitopes, identified as described above, can be used as reagents to assist in the definition of the biochemical nature of these epitopes. Cross-linking studies employing derivatized Fabs can be employed to label amino acid residues in the vicinity of the epitopes. Similarly, the Fabs can be used in protease protection studies to identify those domains of the allergen protein which are shielded from protease degradation by pre-binding of a specific Fab. Experiments employing recombinant monoclonal Fabs as reagents to label or protect from labeling should permit at least partial elucidation of the structures of conformational epitopes.

"Humanized" recombinant Fabs should bind to allergens if injected into a patient and thus prevent the binding of these allergens to native IgE. Since the Fabs cannot interact with the Fcε receptor, the binding of the IgE Fabs to allergen would not be expected to elicit mast cell degranulation. Allergen should be neutralized as it is by protective IgGs.

Anti-idiotypic antibodies directed against the conformational epitope-specific Fabs should resemble the conformation epitopes themselves. Injection of these anti-idiotypic antibodies should induce the production of anti-anti-idiotypic IgGs which would recognize, bind to and inactivate the conformational epitopes. The method through which the anti-idiotypic antibodies would be produced (i.e. animal immunization, "in vitro" immunization or recombinant phage display library) would have to be determined. Similarly, the possibility that the anti-idiotypic antibodies (which

resemble the conformational epitopes) would be recognized by patient IgEs and induce mast cell degranulation needs to be considered.

II. Diagnostic and Therapeutic Procedures Using Modified Allergens.

It is important to administer the modified allergen to an individual (human or animal) to decrease the clinical symptoms of allergic disease by using a method, dosage, and carrier which are effective. Allergen will typically be administered in an appropriate carrier, such as saline or a phosphate saline buffer. Allergen can be administered by injection subcutaneously, intramuscularly, or intraperitoneally (most humans would be treated by subcutaneous injection), by aerosol, inhaled powder, or by ingestion.

Therapy or desensitization with the modified allergens can be used in combination with other therapies, such as allergen-non-specific anti-IgE antibodies to deplete the patient of allergen-specific IgE antibodies (Boulet, et al. (1997) 155:1835-1840; Fahy, et al. (1997) *American J Respir. Crit. Care Med.* 155:1828-1834; Demoly, P. and Bousquet (1997) *J Am J Resp. Crit. Care Med.* 155:1825-1827), or by the pan specific anti-allergy therapy described in U. S. Serial No. 08/090,375 filed June 4, 1998, by M. Caplan and H. Sosin. Therapy with the modified allergen can also be administered in combination with an adjuvant such as IL 12, IL 16, IL 18, Ifn- γ .

The nucleotide molecule encoding the modified allergen can also be administered directly to the patient, for example, in a suitable expression vector such as a plasmid, which is injected directly into the muscle or dermis, or through administration of genetically engineered cells.

In general, effective dosages will be in the picogram to milligram range, more typically microgram to milligram. Treatment will typically be between twice/weekly and once a month, continuing for up to three to five years, although this is highly dependent on the individual patient response.

The modified allergen can also be used as a diagnostic to characterize

the patient's allergies, using techniques such as those described in the examples.

EXAMPLES

Peanut allergy is one of the most common and serious of the immediate hypersensitivity reactions to foods in terms of persistence and severity of reaction. Unlike the clinical symptoms of many other food allergies, the reactions to peanuts are rarely outgrown, therefore, most diagnosed children will have the disease for a lifetime (Sampson, H.A., and Burks, A.W. (1996) *Annu. Rev. Nutr.* 16, 161-77; Bock, S.A. (1985) *J. Pediatr.* 107, 676-680). The majority of cases of fatal food-induced anaphylaxis involve ingestion of peanuts (Sampson et al., (1992) *NEJM* 327, 380-384; Kaminogawa, S. (1996) *Biosci. Biotech. Biochem.* 60, 1749-1756). The only effective therapeutic option currently available for the prevention of a peanut hypersensitivity reaction is food avoidance. Unfortunately, for a ubiquitous food such as a peanut, the possibility of an inadvertent ingestion is great.

The examples described below demonstrate identification, modification, and assessment of allergenicity of the major peanut allergens, Ara h 1, Ara h 2, and Ara h 3. Detailed experimental procedures are included for Example 1. These same procedures were used for Examples 2-5. The nucleotide sequences of Ara h 1, Ara h 2, and Ara h 3, are shown in SEQ ID NOs. 1, 3, and 5, respectively. The amino acid sequences of Ara h 1, Ara h 2, and Ara h 3 are shown in SEQ ID NOs. 2, 4, and 6 respectively.

Example 1: Identification of linear IgE binding epitopes.

Due to the significance of the allergic reaction and the widening use of peanuts as protein extenders in processed foods, there is increasing interest in defining the allergenic proteins and exploring ways to decrease the risk to the peanut-sensitive individual. Various studies over the last several years

have identified the major allergens in peanuts as belonging to different families of seed storage proteins (Burks, et al. (1997) *Eur. J. Biochem.* 245, 334-339; Stanley, et al. (1997) *Arch. Biochem. Biophys.* 342, 244-253). The major peanut allergens Ara h 1, Ara h 2, and Ara h 3 belong to the vicilin, conglutin and glycinin families of seed storage proteins, respectively. These allergens are abundant proteins found in peanuts and are recognized by serum IgE from greater than 95% of peanut sensitive individuals, indicating that they are the major allergens involved in the clinical etiology of this disease (Burks, et al. (1995) *J. Clinical Invest.*, 96, 1715-1721). The genes encoding Ara h 1 (SEQ ID NO. 1), Ara h 2 (SEQ ID NO. 3), and Ara h 3 (SEQ ID NO. 5) and the proteins encoded by these genes (SEQ ID NO. 2, 4, 6) have been isolated and characterized. The following studies were conducted to identify the IgE epitopes of these allergens recognized by a population of peanut hypersensitive patients and a means for modifying their affinity for IgE.

Experimental Procedures

Serum IgE. Serum from 15 patients with documented peanut hypersensitivity reactions (mean age, 25 yrs) was used to determine relative binding affinities between wild type and mutant Ara h 1 synthesized epitopes. The patients had either a positive double-blind, placebo-controlled, food challenge or a convincing history of peanut anaphylaxis (laryngeal edema, severe wheezing, and/or hypotension; Burks, et al. (1988) *J. Pediatr.* 113, 447-451). At least 5 ml of venous blood was drawn from each patient, allowed to clot, and serum was collected. A serum pool from 12 to 15 patients was made by mixing equal aliquots of serum IgE from each patient. The pools were then used in immunoblot analysis.

Peptide synthesis. Individual peptides were synthesized on a derivatized cellulose membrane using 9-fluorenylmethoxycarbonyl (Fmoc) amino acid active esters according to the manufacturer's instructions

(Genosys Biotechnologies, Woodlands, Texas; Fields, G.B. and Noble, R.L. (1990) *Int. J. Peptide Protein Res.* 35, 161-214). Fmoc-amino acids (N-terminal blocked) with protected side chains were coupled in the presence of 1-methyl-2-pyrrolidone to a derivatized cellulose membrane. Following washing with dimethylformamide (DMF), unreacted terminal amino groups were blocked from further reactions by acetylation with acetic anhydride. The N-terminal Fmoc blocking group was then removed by reaction with 20% piperidine and 80% DMF, v/v. The membrane was washed in DMF followed by methanol, the next reactive Fmoc-amino acid was then coupled as before, and the sequence of reactions was repeated with the next amino acid. When peptide synthesis was complete, the side chains were deprotected with a mixture of dichloromethane (DCM), trifluoroacetic acid, and triisobutylsilane (1.0:1.0:0.5), followed by successive washes in DCM, DMF, and methanol. Peptides synthesis reactions were monitored by bromophenol blue color reactions during certain steps of synthesis. Cellulose derivitised membranes and Fmoc-amino acids were supplied by Genosys Biotechnologies. All other chemical were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI) or Fluka (Buchs, Switzerland). Membranes were either probed immediately or stored at -20°C until needed.

IgE binding assays. Cellulose membranes containing synthesized peptides were washed 3 times in Tris-buffered saline (TBS; 136 mM NaCl, 2.7 mM KCl, and 50 mM trizma base pH 8.0) for 10 min at room temperature (RT) and then incubated overnight in blocking buffer: [TBS, 0.05% TweenTM 20; concentrated membrane blocking buffer supplied by Genosys; and sucrose (0.0:1.0:0.5)]. The membrane was then incubated in pooled sera diluted in 1:5 in 20 mM Tris-Cl pH7.5, 150 mM NaCl, and 1% bovine serum albumin overnight at 4°C. Primary antibody was detected with ¹²⁵I-labeled equine anti-human IgE (Kallestad, Chaska, MN).

Quantitation of IgE binding. Relative amounts of IgE binding to individual peptides were determined by a Bio-Rad (Hercules, CA) model GS-700 imaging laser densitometer and quantitated with Bio-Rad molecular analyst software. A background area was scanned and subtracted from the obtained values. Following quantitation, wild type intensities were normalized to a value of one and the mutants were calculated as percentages relative to the wild type.

Synthesis and purification of recombinant Ara h 2 protein. cDNA encoding Ara h 2 was placed in the pET-24b expression vector. The pET-24 expression vector places a 6 x histidine tag at the carboxyl end of the inserted protein. The histidine tag allows the recombinant protein to be purified by affinity purification on a nickel column (HisBind resin). Recombinant Ara h 2 was expressed and purified according to the instructions of the pET system manual. Briefly, expression of the recombinant Ara h 2 was induced in 200 ml cultures of strain BL21(DE3) E. coli with 1 mM IPTG at mid log phase. Cultures were allowed to continue for an additional 3 hours at 36°C. Cells were harvested by centrifugation at 2000 x g for 15 minutes and then lysed in denaturing binding buffer (6 M urea, 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Lysates were cleared by centrifugation at 39,000 x g for 20 minutes followed by filtration through 0.45 micron filters. The cleared lysate was applied to a 10 ml column of HisBind resin, washed with imidazole wash buffer (20 mM imidazole, 6 M urea, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The recombinant Ara h 2 was then released from the column using elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The elution buffer was replaced with phosphate buffered saline by dialysis. The purification of recombinant Ara h 2 was followed by SDS PAGE and immunoblots. Peanut specific serum IgE was used as a primary antibody.

Skin prick tests. The ability of purified native and recombinant Ara h 2 to elicit the IgE mediated degranulation of mast cells was evaluated using prick skin tests in a peanut allergic individual. An individual meeting the criteria for peanut allergy (convincing history or positive double blind placebo controlled food challenge) and a non-allergic control were selected for the testing. Purified native and recombinant Ara h 2 and whole peanut extract (Greer Laboratories, Lenoir, N.C.) were tested. Twenty microliters of the test solution were applied to the forearm of the volunteer and the skin beneath pricked with a sterile needle. Testing was started at the lowest concentration (less than or equal to 1 mg/ml) and increased ten fold each round to the highest concentration or until a positive reaction was observed. Mean diameters of the wheal and erythema were measured and compared to the negative saline control. A positive reaction was defined as a wheal 3mm larger than the negative control. Histamine was used as the positive control.

Results

Identification of the linear IgE-binding epitopes of Ara h 1, Ara h 2 and Ara h 3 allergens. Epitope mapping was performed on the Ara h 1, Ara h 2 and Ara h 3 allergens by synthesizing each of these proteins in 15 amino acid long overlapping peptides that were offset from each other by 8 amino acids. The peptides were then probed with a pool of serum IgE from 15 patients with documented peanut hypersensitivity. This analysis resulted in multiple IgE binding regions being identified for each allergen. The exact position of each IgE binding epitope was then determined by re-synthesizing these IgE reactive regions as 10 or 15 amino acid long peptides that were offset from each other by two amino acids. These peptides were probed with the same pool of serum IgE from peanut sensitive patients as used before. An example of this procedure for each of the peanut allergens is shown in Fig. 1-3 (Ara h 1 - Fig. 1; Ara h 2 - Fig. 2; Ara h 3 - Fig. 3). This analysis

revealed that there were 23 linear IgE binding epitopes on Ara h 1, 10 epitopes on Ara h 2, and 4 epitopes on Ara h 3.

In an effort to determine which, if any, of the epitopes were recognized by the majority of patients with peanut hypersensitivity, each set of epitopes identified for the peanut allergens were synthesized and then probed individually with serum IgE from 10 different patients. All of the patient sera tested recognized multiple epitopes.

Table 1 shows the amino acid sequence and position of each epitope within the Ara h 1 protein of all 23 IgE binding epitopes mapped to this molecule. Table 2 shows the amino acid sequence and position of each epitope within the Ara h 2 protein of all 10 IgE binding epitopes mapped to this molecule. Table 3 shows the amino acid sequence and position of each epitope within the Ara h 3 protein of all 4 IgE binding epitopes mapped to this molecule.

Four epitopes of the Ara h 1 allergen (peptides 1,3,4,17 of Table 1), three epitopes of the Ara h 2 allergen (peptides 3,6,7 of Table 2), and 1 epitope of the Ara h 3 allergen (peptide 2 of Table 3) were immunodominant.

Table 1. Ara h I IgE Binding Epitopes

| EPITOPE | AA SEQUENCE | POSITION |
|----------------|--------------------|-----------------|
| 1 | <u>AKSSPYQKKT</u> | 25-34 |
| 2 | <u>QEPDDLKOKA</u> | 48-57 |
| 3 | <u>LEYDPRLVYD</u> | 65-74 |
| 4 | <u>GERTRGROPG</u> | 89-98 |
| 5 | <u>PGDYDDDRRQ</u> | 97-106 |
| 6 | <u>PRREEGGRWG</u> | 107-116 |
| 7 | <u>REEREEDWRQP</u> | 123-132 |
| 8 | <u>EDWRRPSHQQ</u> | 134-143 |
| 9 | <u>QPRKIRPEGR</u> | 143-152 |
| 10 | <u>TPGQFEDFFP</u> | 294-303 |
| 11 | <u>SYLQEFNRNT</u> | 311-320 |
| 12 | <u>FNAEFNEIRR</u> | 325-334 |
| 13 | <u>EQEERGQRRW</u> | 344-353 |
| 14 | <u>DITNPINLRE</u> | 393-402 |
| 15 | <u>NNFGKLFVK</u> | 409-418 |
| 16 | <u>GTGNLELVAV</u> | 461-470 |
| 17 | <u>RRYTARLKEG</u> | 498-507 |
| 18 | <u>ELHLLGFGIN</u> | 525-534 |
| 19 | <u>HRIFLAGDKD</u> | 539-548 |
| 20 | <u>IDQIEKQAKD</u> | 551-560 |
| 21 | <u>KDLAFPGSGE</u> | 559-568 |
| 22 | <u>KESHFVSARP</u> | 578-587 |
| 23 | <u>PEKESPEKED</u> | 597-606 |

The underlined portions of each peptide are the smallest IgE binding sequences as determined by this analysis. All of these sequences can be found in SEQ ID NO 2.

Table 2. Ara h 2 IgE Binding Epitopes

| EPITOPE | AA SEQUENCE | POSITION |
|---------|-------------------|----------|
| 1 | <u>HASARQQWEL</u> | 15-24 |
| 2 | <u>QWELQDRLRC</u> | 21-30 |
| 3 | <u>DRRCQSOLER</u> | 27-36 |
| 4 | <u>LRPCEQHLMQ</u> | 39-48 |
| 5 | <u>KIQRDEDSYE</u> | 49-58 |
| 6 | <u>YERDPYSPSQ</u> | 57-66 |
| 7 | <u>SQDPYSPSPY</u> | 65-74 |
| 8 | <u>DRLOGRQQEQ</u> | 115-124 |
| 9 | <u>KRELRLNPQQ</u> | 127-136 |
| 10 | <u>QRCDLDVESG</u> | 143-152 |

The underlined portions of each peptide are the smallest IgE binding sequences as determined by this analysis. All of these sequences can be found in SEQ ID NO 4.

Table 3. Ara h 3 IgE Binding Epitopes

| EPITOPE | AA SEQUENCE | POSITION |
|---------|------------------------|----------|
| 1 | <u>IETWNPNNQEFECAG</u> | 33-47 |
| 2 | <u>GNIFSGFTPEFLEQA</u> | 240-254 |
| 3 | <u>VTVRGGLRILSPDRK</u> | 279-293 |
| 4 | <u>DEDEYDEEDRRRG</u> | 303-317 |

The underlined portions of each peptide are the smallest IgE binding sequences as determined by this analysis. All of these sequences can be found in SEQ ID NO 6.

Example 2 : Modification of peanut allergens to decrease allergenicity.

The major linear IgE binding epitopes of the peanut allergens were mapped using overlapping peptides synthesized on an activated cellulose membrane and pooled serum IgE from 15 peanut sensitive patients, as described in Example 1. The size of the epitopes ranged from six to fifteen amino acids in length. The amino acids essential to IgE binding in each of the epitopes were determined by synthesizing duplicate peptides with single amino acid changes at each position. These peptides were then probed with pooled serum IgE from 15 patients with peanut hypersensitivity to determine if the changes affected peanut-specific IgE binding. For example, epitope 9 in Table 1 was synthesized with an alanine or methionine residue substituted for one of the amino acids and probed. The following amino acids were substituted (first letter is the one-letter amino acid code for the residue normally at the position, the residue number, followed by the amino acid that was substituted for this residue; the numbers indicate the position of each residue in the Ara h 1 protein, SEQ ID NO. 2): Q143A, P144A; R145A; K146A; I147A; R148A; P149A; E150A; G151A; R152A; Q143M; P144M; R145M; K146M; I147M; R148M; P149M; E150M; G151M; R152M. The immunoblot strip containing the wild-type and mutated peptides of epitope 9 showed that binding of pooled serum IgE to individual peptides was dramatically reduced when either alanine or methionine was substituted for each of the amino acids at positions 144, 145, and 147-150 of Ara h 1 shown in SEQ ID NO. 2. Changes at positions 144, 145, 147, and 148 of Ara h 1 shown in SEQ ID NO. 2 had the most dramatic effect when methionine was substituted for the wild-type amino acid, resulting in less than 1% of peanut specific IgE binding to these peptides. In contrast, the substitution of an alanine for arginine at position 152 of Ara h 1 shown in SEQ ID NO. 2 resulted in increased IgE binding. The remaining Ara h 1 epitopes, and the Ara h 2 and Ara h 3 epitopes, were tested in the same manner and the

intensity of IgE binding to each spot was determined as a percentage of IgE binding to the wild-type peptide. Any amino acid substitution that resulted in less than 1% of IgE binding when compared to the wild type peptide was noted and is indicated in Tables 4-6. Table 4 shows the amino acids that were determined to be critical to IgE binding in each of the Ara h 1 epitopes. Table 5 shows the amino acids that were determined to be critical to IgE binding in each of the Ara h 2 epitopes. Table 6 shows the amino acids that were determined to be critical to IgE binding in each of the Ara h 3 epitopes. This analysis indicated that each epitope could be mutated to a non-IgE binding-peptide by the substitution of a single amino acid residue.

The results discussed above for Ara h 1, Ara h 2, and Ara h 3 demonstrate that once an IgE binding site has been identified, it is possible to reduce IgE binding to this site by altering a single amino acid of the epitope. The observation that alteration of a single amino acid leads to the loss of IgE binding in a population of peanut-sensitive individuals is significant because it suggests that while each patient may display a polyclonal IgE reaction to a particular allergen, IgE from different patients that recognize the same epitope must interact with that epitope in a similar fashion. Besides finding that many epitopes contained more than one residue critical for IgE binding, it was also determined that more than one residue type (ala or met) could be substituted at certain positions in an epitope with similar results. This allows for the design of a hypoallergenic protein that would be effective at blunting allergic reactions for a population of peanut sensitive individuals. Furthermore, the creation of a plant producing a peanut where the IgE binding epitopes of the major allergens have been removed should prevent the development of peanut hypersensitivity in individuals genetically predisposed to this food allergy.

Table 4: Amino Acids Critical to IgE Binding of Ara h 1

| EPITOPE | AA SEQUENCE | POSITION |
|---------|---|----------|
| 1 | AKSSPYQ K KT | 25-34 |
| 2 | QEP DDL KQKA | 48-57 |
| 3 | LEY D PRLV YD | 65-74 |
| 4 | GER T RGR Q PG | 89-98 |
| 5 | PGDYDD DRR Q | 97-106 |
| 6 | PRREE G GRWG | 107-116 |
| 7 | REREED W R Q P | 123-132 |
| 8 | EDW R RRPS H QQ | 134-143 |
| 9 | Q PR K IRPEGR | 143-152 |
| 10 | T P G Q FED F FP | 294-303 |
| 11 | S YL Q EFSRNT | 311-320 |
| 12 | FNA E FNEIRR | 325-334 |
| 13 | EQEER G QRRW | 344-353 |
| 14 | DIT N PIN L RE | 393-402 |
| 15 | NNFG K LFEVK | 409-418 |
| 17 | R RYTARLKEG | 498-507 |
| 18 | EL H LL G FGIN | 525-534 |
| 19 | HRIFLAGD K D | 539-548 |
| 20 | IDQ I E K Q A K D | 551-560 |
| 21 | KDLA F PGSGE | 559-568 |
| 22 | KESHFV S ARP | 578-587 |

Note. The Ara h 1 IgE binding epitopes are indicated as the single letter amino acid code. The position of each peptide with respect to the Ara h 1 protein is indicated in the right hand column. The amino acids that, when altered, lead to loss of IgE binding are shown as the bold, underlined residues. Epitopes 16 and 23 were not included in this study because they were recognized by a single patient who was no longer available to the study. All of these sequences can be found in SEQ ID NO 2.

Table 5. Amino Acids Critical to IgE Binding of Ara h 2

| EPITOPE | AA SEQUENCE | POSITION |
|---------|---|----------|
| 1 | HASAR <u>Q</u> <u>Q</u> WEL | 15-24 |
| 2 | QWEL <u>Q</u> <u>G</u> DRRC | 21-30 |
| 3 | <u>D</u> <u>R</u> <u>R</u> C <u>Q</u> <u>S</u> <u>Q</u> <u>L</u> <u>E</u> <u>R</u> | 27-36 |
| 4 | <u>L</u> <u>R</u> <u>P</u> <u>C</u> <u>E</u> <u>Q</u> <u>H</u> <u>L</u> <u>M</u> <u>Q</u> | 39-48 |
| 5 | <u>K</u> <u>I</u> <u>Q</u> <u>R</u> <u>D</u> <u>E</u> <u>D</u> <u>S</u> <u>Y</u> <u>E</u> | 49-58 |
| 6 | YER <u>D</u> <u>P</u> <u>Y</u> <u>S</u> <u>P</u> <u>S</u> <u>Q</u> | 57-66 |
| 7 | <u>S</u> <u>Q</u> <u>D</u> <u>P</u> <u>Y</u> <u>S</u> <u>P</u> <u>S</u> <u>P</u> <u>Y</u> | 65-74 |
| 8 | DR <u>L</u> <u>Q</u> <u>G</u> <u>R</u> <u>Q</u> <u>Q</u> <u>E</u> <u>Q</u> | 115-124 |
| 9 | <u>K</u> <u>R</u> <u>E</u> <u>L</u> <u>R</u> <u>N</u> <u>L</u> <u>P</u> <u>Q</u> <u>Q</u> | 127-136 |
| 10 | Q <u>R</u> <u>C</u> <u>D</u> <u>L</u> <u>D</u> <u>V</u> <u>E</u> <u>S</u> <u>G</u> | 143-152 |

Note. The Ara h 2 IgE binding epitopes are indicated as the single letter amino acid code. The position of each peptide with respect to the Ara h 2 protein is indicated in the right hand column. The amino acids that, when altered, lead to loss of IgE binding are shown as the bold, underlined residues. All of these sequences can be found in SEQ ID NO 4.

Table 6. Amino Acids Critical to IgE-Binding of Ara h 3.

| EPITOPE | AA SEQUENCE | POSITION |
|---------|--|----------|
| 1 | IETW <u>N</u> <u>P</u> <u>N</u> <u>N</u> QEFECAG | 33-47 |
| 2 | GN <u>I</u> <u>F</u> <u>S</u> <u>G</u> <u>F</u> <u>T</u> <u>P</u> <u>E</u> <u>F</u> <u>L</u> <u>E</u> <u>Q</u> <u>A</u> | 240-254 |
| 3 | VT <u>V</u> <u>R</u> <u>G</u> <u>G</u> <u>L</u> <u>R</u> <u>I</u> <u>L</u> <u>S</u> <u>P</u> <u>D</u> <u>R</u> <u>K</u> | 279-293 |
| 4 | DEDE <u>Y</u> <u>E</u> <u>Y</u> <u>D</u> <u>E</u> <u>E</u> <u>D</u> <u>R</u> <u>R</u> <u>R</u> <u>G</u> | 303-317 |

Note. The Ara h 3 IgE binding epitopes are indicated as the single letter amino acid code. The position of each peptide with respect to the Ara h 3 protein is indicated in the right hand column. The amino acids that, when altered, lead to loss of IgE binding are shown as the bold, underlined. All of these sequences can be found in SEQ ID NO 6.

Example 3: A Modified Ara h 2 Protein Binds less IgE But Similar Amounts of IgG.

In order to determine the effect of changes to multiple epitopes within the context of the intact allergen, four epitopes (including the three immunodominant epitopes) of the Ara h 2 allergen were mutagenized and the protein produced recombinantly. The amino acids at position 20, 31, 60, and 67 of the Ara h 2 protein (shown in SEQ ID NO. 4 and Figure 4A) were changed to alanine by mutagenizing the gene encoding this protein by standard techniques. These residues are located in epitopes 1, 3, 6, and 7 and represent amino acids critical to IgE binding that were determined in Example 2. The modified and wild-type versions of this protein were produced and immunoblot analysis performed using serum from peanut sensitive patients. These results showed that the modified version of this allergen bound significantly less IgE than the wild type version of these recombinant proteins (Figure 4B) but bound similar amounts of IgG.

Example 4: A modified Ara h 2 protein retains the ability to stimulate T-cells to proliferate.

The modified recombinant Ara h 2 protein described in Example 3 was used in T-cell proliferation assays to determine if it retained the ability to activate T cells from peanut sensitive individuals. Proliferation assays were performed on T-cell lines grown in short-term culture developed from six peanut sensitive patients. T-cells lines were stimulated with either 50 μ g of crude peanut extract, 10 μ g of native Ara h 2, 10 μ g of recombinant wild-type Ara h2, or 10 μ g of modified recombinant Ara h 2 protein and the amount of 3H-thymidine determined for each cell line. Results were expressed as the average stimulation index (SI) which reflected the fold increase in 3H-thymidine incorporation exhibited by cells challenged with allergen when compared with media treated controls (Figure 5).

Example 5: A Modified Ara h 2 Protein Elicits a Smaller Wheal and Flare in Skin Prick Tests of a Peanut Sensitive Individual.

5 The modified recombinant Ara h 2 protein described in Example 3 and the wild type version of this recombinant protein were used in a skin prick test of a peanut sensitive individual. Ten micrograms of these proteins were applied separately to the forearm of a peanut sensitive individual, the skin pricked with a sterile needle, and 10 minutes later any wheal and flare that developed was measured. The wheal and flare produced by the wild-type Ara h 2 protein (8 mm X 7 mm) was approximately twice as large as that produced by the modified Ara h 2 protein (4 mm X 3mm). A control subject (no peanut hypersensitivity) tested with the same proteins had no visible wheal and flare but, as expected, gave positive results when challenged with histamine. In addition, the test subject gave no positive results when tested with PBS alone. These results indicate that an allergen with only 40% of its IgE binding epitopes modified (4/10) can give measurable reduction in reactivity in an *in vivo* test of a peanut sensitive patient.

15 These same techniques can be used with the other known peanut allergens, Ara h 1 (SEQ ID NO 1 and 2), Ara h 3 (SEQ ID NO. 5 and 6), or any other allergen.

20 Modifications and variations of the methods and materials described herein will be obvious to those skilled in the art. Such modifications and variations are intended to come within the scope of the appended claims.

We claim:

1. A method of making a modified allergen which is less reactive with IgE comprising
 - (a) identifying IgE binding sites in an allergen;
 - (b) modifying the allergen by mutating at least one amino acid in an IgE binding site or reacting the allergen with a compound blocking binding to at least one amino acid in an IgE binding site;
 - (c) screening for IgE binding to the modified allergen using serum or antibodies from a pooled patient population and screening for activation of T cells; and
 - (d) selecting the modified allergens which have decreased binding to IgE as compared to the unmodified allergen and which activate T cells.
2. The method of claim 1 further comprising screening for binding of the modified allergen for binding to IgG and selecting the modified allergens which have decreased binding to IgE, activate T cells and bind to IgG.
3. The method of claim 1 wherein the modified allergen is mutated in the center of one or more of the IgE binding sites.
4. The method of claim 1 wherein the modified allergen is mutated by substituting a hydrophobic amino acid in the center of one or more of the IgE binding sites with a neutral or hydrophilic amino acid.
5. The method of claim 1 wherein binding of IgE to the modified allergen is blocked by reaction of a compound with at least one amino acid present in an IgE binding site.
6. The method of claim 5 wherein binding of IgE is blocked by reaction of the allergen with an antibody which blocks binding to one or more IgE sites but allows the allergen to still activate T cells.
7. The method of claim 1 wherein the modified allergen is a portion of a protein.

8. The method of claim 1 wherein the modified allergen is formulated with an adjuvant selected from the group consisting of IL 12, IL 16, IL 18, Ifn- γ or immune stimulatory sequences.
9. The method of claim 1 wherein the modified allergen is screened for initiation of a T cell helper 1 response.
10. The method of claim 1 wherein the modified allergen is made in a recombinant host selected from the group consisting of plants, animals, bacteria, yeast, fungi, and insect cells.
11. The method of claim 1 wherein the modified allergen is made in cells using site specific mutation.
12. The method of claim 1 wherein the modified allergen is made from a peanut allergen selected from the group consisting of Ara h 1, Ara h 2, and Ara h 3.
13. The method of claim 1 wherein the modified allergen is based on a protein obtained from a source selected from the group consisting of legumes, milks, grains, eggs, fish, crustaceans, mollusks, insects, molds, dust, grasses, trees, weeds, mammals, birds, and natural latexes.
14. A modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change or having at least one amino acid bound by a compound so that the site no longer binds IgE, wherein the modified allergen activates T cells.
15. The modified allergen of claim 14 wherein the modified allergen binds IgG.
16. The modified allergen of claim 14 made by the process of
 - (a) identifying one or more IgE binding sites in an allergen;
 - (b) mutating at least one amino acid in an IgE binding site;
 - (c) screening for IgE binding to the mutated allergen and activation of T cells by the mutated allergen; and

(d) selecting the modified allergens with decreased binding to IgE which activate T cells.

17. The modified allergen of claim 14 wherein the modified allergen is mutated in the center of one or more of the IgE binding sites.

18. The modified allergen of claim 14 wherein the modified allergen is mutated by substituting a hydrophobic amino acid in the center of one or more of the IgE binding sites with a neutral or hydrophilic amino acid.

19. The modified allergen of claim 14 wherein binding of IgE is blocked by reaction of a compound with at least one amino acid present in an IgE binding site.

20. The modified allergen of claim 19 wherein binding of IgE is blocked by reaction of the allergen with an antibody which blocks binding to one or more IgE sites but allows the allergen to still activate T cells.

21. The modified allergen of Claim 20 wherein the modified allergen does not have significantly altered or decreased IgG binding capacity.

22. The modified allergen of claim 14 which initiates a T cell helper 1 response.

23. The modified allergen of claim 14 wherein the allergen is a portion of a protein.

24. The modified allergen of claim 14 wherein the modified allergen is formulated with an adjuvant selected from the group consisting of IL 12, IL 16, IL 18, Ifn- γ and immune stimulatory sequences.

25. The modified allergen of claim 14 wherein the modified allergen is made in a transgenic plant or animal.

26. The modified allergen of claim 14 expressed in a recombinant host selected from the group consisting of plants and animals.

27. The modified allergen of claim 17 expressed in a recombinant host selected from the group consisting of bacteria, yeast, fungi, and insect cells.

28. The modified allergen of claim 14 wherein the modified allergen is based on a protein obtained from a source selected from the group consisting of legumes, milks, grains, eggs, fish, crustaceans, mollusks, insects, molds, dust, grasses, trees, weeds, mammals, birds, and natural latexes.
29. The modified allergen of claim 14 wherein the modified allergen is made from a peanut allergen selected from the group consisting of Ara h 1, Ara h 2, and Ara h 3.
30. A nucleotide molecule encoding a modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change so that the site no longer binds IgE, but wherein the modified allergen activates T cells, as defined by any of claims 14, 15, 16, 17, 18, 21 or 22.
31. The molecule of claim 30 in a vector for expression in a recombinant host.
32. A nucleotide molecule for causing a site specific mutation in a gene encoding a protein which yields a modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change so that the site no longer binds IgE, but wherein the modified allergen activates T cells.
33. A transgenic plant expressing a modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change so that the site no longer binds IgE, but wherein the modified allergen activates T cells, as defined by any of claims 14, 15, 16, 17, 18, 19, 20, 21, 22, or 23.
34. A transgenic animal expressing a modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change so that the site no longer binds IgE, but wherein the modified allergen activates T cells, as defined by any of claims 14, 15, 16, 17, 18, 19, 20, 21, 22, or 23.

35. A compound selectively binding to at least one amino acid in an IgE binding site of an allergen, wherein the site no longer binds IgE, but wherein the allergen is able to activate T cells, wherein the compound is obtained using a combinatorial library or combinatorial chemistry and screening for reaction with the allergen to produce bound allergen, followed by testing of the bound allergen for binding to IgE and activation of T cells.

36. A method to treat an individual to reduce the clinical response to an allergen comprising administering to the individual a modified allergen which is less reactive with IgE comprising at least one IgE binding site present in the allergen modified by at least one amino acid change or having at least one amino acid bound by a compound so that the site no longer binds IgE, wherein the modified allergen activates T cells, as defined by any of claims 14-29, in an amount and for a time sufficient to reduce the allergic reaction to the unmodified allergen.

METHODS AND REAGENTS FOR DECREASING CLINICAL REACTION TO ALLERGY

Abstract

It has been determined that allergens, which are characterized by both humoral (IgE) and cellular (T cell) binding sites, can be modified to be less allergenic by modifying the IgE binding sites. The IgE binding sites can be converted to non-IgE binding sites by masking the site with a compound that prevents IgE binding or by altering as little as a single amino acid within the protein, most typically a hydrophobic residue towards the center of the IgE-binding epitope, to eliminate IgE binding. The method allows the protein to be altered as minimally as possible, other than within the IgE-binding sites, while retaining the ability of the protein to activate T cells, and, in some embodiments by not significantly altering or decreasing IgG binding capacity. The examples use peanut allergens to demonstrate alteration of IgE binding sites. The critical amino acids within each of the IgE binding epitopes of the peanut protein that are important to immunoglobulin binding have been determined. Substitution of even a single amino acid within each of the epitopes led to loss of IgE binding. Although the epitopes shared no common amino acid sequence motif, the hydrophobic residues located in the center of the epitope appeared to be most critical to IgE binding.

SEQUENCE LISTING

<110> Bannon, Gary A

Burks, A Wesley

Sampson, Hugh

Sosin, Howard

<120> Methods and Reagents for Decreasing Clinical Reaction
to Allergy

<130> HS102

<140>

<141>

<150> PCT/US96/15222

<151> 1996-09-23

<150> 60/074590

<151> 1998-02-13

<150> 60/074624

<151> 1998-02-13

<150> 60/074633

<151> 1998-02-13

<160> 6

<170> PatentIn Ver. 2.0

<210> 1

<211> 1930

<212> DNA

<213> Peanut

<400> 1

```

aataatcata tatattcatc aatcatctat ataagtagta gcaggagcaa tgagagggag 60
ggttttctcca ctgatgctgt tgctagggat ccttgctcctg gcttcagttt ctgcaacgca 120
tgccaagtca tcaccttacc agaagaaaac agagaacccc tgcgcccaga ggtgcctcca 180
gagttgtcaa caggaaccgg atgacttgaa gcaaaaggca tgcgagtctc gctgcaccaa 240
gctcgagtat gatcctcggt gtgtctatga tctctgagga cactctggca ccaccaacca 300
acgttccccct ccaggggagc ggacacgtgg ccgccaaccc ggagactacg atgatgaccg 360
ccgtcaaccc cgaagagagg aaggaggccg atggggacca gctggaccga gggagcgtga 420
aagagaagaa gactggagac aaccaagaga agattggagg cgaccaagtc atcagcagcc 480
acggaaaata aggccccaag gaagagaagg agaacaagag tggggaacac caggtagcca 540
tgtgagggaa gaaacatctc ggaacaaccc tttctacttc ccgtcaaggc ggtttagcac 600
ccgctacggg aaccaaaacg gtaggatccg ggtcctgcag aggtttgacc aaaggtcaag 660
gcagtttcag aatctccaga atcaccgtat tgtgcagatc gaggccaaac ctaacactct 720
tgttcttccc aagcacgctg atgctgataa catccttggt atccagcaag ggcaagccac 780
cgtgaccgta gcaaattggc ataacagaaa gagctttaat cttgacgagg gccatgcact 840
cagaatccca tccggtttca tttctacat cttgaaccgc catgacaacc agaacctcag 900
agtagctaaa atctccatgc ccgttaacac acccggccag tttgaggatt tcttcccggc 960
gagcagccga gaccaatcat cctacttgca gggcttcagc aggaatacgt tggaggccgc 1020
cttcaatgcg gaattcaatg agatacggag ggtgctgtta gaagagaatg caggaggtga 1080
gcaagaggag agagggcaga ggcgatggag tactcggagt agtgagaaca atgaaggagt 1140
gatagtcaaa gtgtcaaagg agcacgttga agaacttact aagcacgcta aatccgtctc 1200
aaagaaaggc tccgaagaag agggagatat caccaaccca atcaacttga gagaaggcga 1260
gcccgatctt tctaacaact ttgggaagtt atttgaggtg aagccagaca agaagaaccc 1320
ccagcttcag gacctggaca tgatgctcac ctgtgtagag atcaaagaag gagctttgat 1380

```

gctccacac ttcaactcaa aggccatggt tatcgtcgtc gtcaacaaag gaactggaaa 1440
 ccttgaactc gtggctgtaa gaaaagagca acaacagagg ggacggcggg aagaagagga 1500
 ggacgaagac gaagaagagg agggaagtaa cagagaggtg cgtaggtaca cagcgagggt 1560
 gaaggaaggc gatgtgttca tcatgccagc agctcatcca gtagccatca acgcttcctc 1620
 cgaactccat ctgcttggct tcggtatcaa cgctgaaaac aaccacagaa tcttccttgc 1680
 aggtgataag gacaatgtga tagaccagat agagaagcaa gcgaaggatt tagcattccc 1740
 tgggtcgggt gaacaagttg agaagctcat caaaaaccag aaggaatctc actttgtgag 1800
 tgctcgtcct caatctcaat ctcaatctcc gtcgtctcct gagaaagagt ctcttgagaa 1860
 agaggatcaa gaggaggaaa accaaggagg gaaggggtcca ctcctttcaa ttttgaaggc 1920
 ttttaactga 1930

<210> 2

<211> 626

<212> PRT

<213> Peanut

<220>

<221> PEPTIDE

<222> (25) .. (34)

<223> peptide 1

<220>

<221> PEPTIDE

<222> (48) .. (57)

<223> peptide 2

<220>

<221> PEPTIDE

<222> (65) .. (74)

<223> peptide 3

<220>

<221> PEPTIDE

<222> (89) .. (98)

<223> peptide 4

<220>

<221> PEPTIDE

<222> (97) .. (106)

<223> peptide 5

<220>

<221> PEPTIDE

<222> (107) .. (116)

<223> peptide 6

<220>

<221> PEPTIDE

<222> (123) .. (132)

<223> peptide 7

<220>

<221> PEPTIDE

<222> (134) .. (143)

<223> peptide 8

<220>

<221> PEPTIDE

<222> (143) .. (152)

<223> peptide 9

<220>

<221> PEPTIDE

<222> (294) .. (303)

<223> peptide 10

<220>

<221> PEPTIDE

<222> (311) .. (320)

<223> peptide 11

<220>

<221> PEPTIDE

<222> (325) .. (334)

<223> peptide 12

<220>

<221> PEPTIDE

<222> (344) .. (353)

<223> peptide 13

<220>

<221> PEPTIDE

<222> (393) .. (402)

<223> peptide 14

<220>

<221> PEPTIDE

<222> (409) .. (418)

<223> peptide 15

<220>

<221> PEPTIDE

<222> (461) .. (470)

<223> peptide 16

<220>

<221> PEPTIDE

<222> (498) .. (507)

<223> peptide 17

<220>

<221> PEPTIDE

<222> (525) .. (534)

<223> peptide 18

<220>

<221> PEPTIDE

<222> (539) .. (548)

<223> peptide 19

<220>

<221> PEPTIDE

<222> (551) .. (560)

<223> peptide 20

<220>

<221> PEPTIDE

<222> (559) .. (568)

<223> peptide 21

<220>

<221> PEPTIDE

<222> (578) .. (587)

<223> peptide 22

<220>

<221> PEPTIDE

<222> (597) .. (606)

<223> peptide 23

<400> 2

Met Arg Gly Arg Val Ser Pro Leu Met Leu Leu Leu Gly Ile Leu Val
1 5 10 15

Leu Ala Ser Val Ser Ala Thr His Ala Lys Ser Ser Pro Tyr Gln Lys
20 25 30

Lys Thr Glu Asn Pro Cys Ala Gln Arg Cys Leu Gln Ser Cys Gln Gln
35 40 45

Glu Pro Asp Asp Leu Lys Gln Lys Ala Cys Glu Ser Arg Cys Thr Lys
50 55 60

Leu Glu Tyr Asp Pro Arg Cys Val Tyr Asp Pro Arg Gly His Thr Gly
65 70 75 80

Thr Thr Asn Gln Arg Ser Pro Pro Gly Glu Arg Thr Arg Gly Arg Gln
85 90 95

Pro Gly Asp Tyr Asp Asp Asp Arg Arg Gln Pro Arg Arg Glu Glu Gly
 100 105 110

Gly Arg Trp Gly Pro Ala Gly Pro Arg Glu Arg Glu Arg Glu Glu Asp
 115 120 125

Trp Arg Gln Pro Arg Glu Asp Trp Arg Arg Pro Ser His Gln Gln Pro
 130 135 140

Arg Lys Ile Arg Pro Glu Gly Arg Glu Gly Glu Gln Glu Trp Gly Thr
 145 150 155 160

Pro Gly Ser His Val Arg Glu Glu Thr Ser Arg Asn Asn Pro Phe Tyr
 165 170 175

Phe Pro Ser Arg Arg Phe Ser Thr Arg Tyr Gly Asn Gln Asn Gly Arg
 180 185 190

Ile Arg Val Leu Gln Arg Phe Asp Gln Arg Ser Arg Gln Phe Gln Asn
 195 200 205

Leu Gln Asn His Arg Ile Val Gln Ile Glu Ala Lys Pro Asn Thr Leu
 210 215 220

Val Leu Pro Lys His Ala Asp Ala Asp Asn Ile Leu Val Ile Gln Gln
 225 230 235 240

Gly Gln Ala Thr Val Thr Val Ala Asn Gly Asn Asn Arg Lys Ser Phe
 245 250 255

Asn Leu Asp Glu Gly His Ala Leu Arg Ile Pro Ser Gly Phe Ile Ser
 260 265 270

Tyr Ile Leu Asn Arg His Asp Asn Gln Asn Leu Arg Val Ala Lys Ile
 275 280 285

Ser Met Pro Val Asn Thr Pro Gly Gln Phe Glu Asp Phe Phe Pro Ala
 290 295 300

Ser Ser Arg Asp Gln Ser Ser Tyr Leu Gln Gly Phe Ser Arg Asn Thr
 305 310 315 320

Leu Glu Ala Ala Phe Asn Ala Glu Phe Asn Glu Ile Arg Arg Val Leu
 325 330 335

Leu Glu Glu Asn Ala Gly Gly Glu Gln Glu Glu Arg Gly Gln Arg Arg
 340 345 350

Trp Ser Thr Arg Ser Ser Glu Asn Asn Glu Gly Val Ile Val Lys Val
 355 360 365

Ser Lys Glu His Val Glu Glu Leu Thr Lys His Ala Lys Ser Val Ser
 370 375 380

Lys Lys Gly Ser Glu Glu Glu Gly Asp Ile Thr Asn Pro Ile Asn Leu
 385 390 395 400

Arg Glu Gly Glu Pro Asp Leu Ser Asn Asn Phe Gly Lys Leu Phe Glu
 405 410 415

Val Lys Pro Asp Lys Lys Asn Pro Gln Leu Gln Asp Leu Asp Met Met
 420 425 430

Leu Thr Cys Val Glu Ile Lys Glu Gly Ala Leu Met Leu Pro His Phe
 435 440 445

Asn Ser Lys Ala Met Val Ile Val Val Val Asn Lys Gly Thr Gly Asn
 450 455 460

Leu Glu Leu Val Ala Val Arg Lys Glu Gln Gln Gln Arg Gly Arg Arg
 465 470 475 480

Glu Glu Glu Glu Asp Glu Asp Glu Glu Glu Glu Gly Ser Asn Arg Glu
 485 490 495

Val Arg Arg Tyr Thr Ala Arg Leu Lys Glu Gly Asp Val Phe Ile Met
 500 505 510

Pro Ala Ala His Pro Val Ala Ile Asn Ala Ser Ser Glu Leu His Leu
 515 520 525

Leu Gly Phe Gly Ile Asn Ala Glu Asn Asn His Arg Ile Phe Leu Ala
 530 535 540

Gly Asp Lys Asp Asn Val Ile Asp Gln Ile Glu Lys Gln Ala Lys Asp
 545 550 555 560

Leu Ala Phe Pro Gly Ser Gly Glu Gln Val Glu Lys Leu Ile Lys Asn
 565 570 575

Gln Lys Glu Ser His Phe Val Ser Ala Arg Pro Gln Ser Gln Ser Gln
 580 585 590

Ser Pro Ser Ser Pro Glu Lys Glu Ser Pro Glu Lys Glu Asp Gln Glu
 595 600 605

Glu Glu Asn Gln Gly Gly Lys Gly Pro Leu Leu Ser Ile Leu Lys Ala
 610 615 620

Phe Asn
 625

<210> 3

<211> 474

<212> DNA

<213> Peanut

<400> 3

ctcaccatac tagtagccct cgccttttct ctctctgctg cccacgcatac tgcgaggcag 60
 cagtgggaac tccaaggaga cagaagatgc cagagccagc tcgagagggc gaacctgagg 120
 ccctgcgagc aacatctcat gcagaagatc caacgtgacg aggattcata tgaacgggac 180
 ccgtacagcc ctagtcagga tccgtacagc cctagtccat atgatcggag aggcgctgga 240
 tcctctcagc accaagagag gtgttgcaat gagctgaacg agtttgagaa caaccaaagg 300
 tgcattgtgc aggcattgca acagatcatg gagaaccaga gcgatagggt gcaggggagg 360
 caacaggagc aacagttcaa gagggagctc aggaacttgc ctcaacagtg cggccttagg 420
 gcaccacagc gttgcgactt ggacgtcgaa agtggcggca gagacagata ctaa 474

<210> 4

<211> 157

<212> PRT

<213> Peanut

<220>

<221> PEPTIDE

<222> (15) .. (24)

<223> peptide 1

<220>

<221> PEPTIDE

<222> (21) .. (30)

<223> peptide 2

<220>

<221> PEPTIDE

<222> (27) .. (36)

<223> peptide 3

<220>

<221> PEPTIDE

<222> (39) .. (48)

<223> peptide 4

<220>

<221> PEPTIDE

<222> (49) .. (58)

<223> peptide 5

<220>

<221> PEPTIDE

<222> (57)..(66)

<223> peptide 6

<220>

<221> PEPTIDE

<222> (65)..(74)

<223> peptide 7

<220>

<221> PEPTIDE

<222> (115)..(124)

<223> peptide 8

<220>

<221> PEPTIDE

<222> (127)..(136)

<223> peptide 9

<220>

<221> PEPTIDE

<222> (143)..(152)

<223> peptide 10

<400> 4

Leu Thr Ile Leu Val Ala Leu Ala Leu Phe Leu Leu Ala Ala His Ala

1

5

10

15

Ser Ala Arg Gln Gln Trp Glu Leu Gln Gly Asp Arg Arg Cys Gln Ser

20

25

30

Gln Leu Glu Arg Ala Asn Leu Arg Pro Cys Glu Gln His Leu Met Gln
 35 40 45

Lys Ile Gln Arg Asp Glu Asp Ser Tyr Glu Arg Asp Pro Tyr Ser Pro
 50 55 60

Ser Gln Asp Pro Tyr Ser Pro Ser Pro Tyr Asp Arg Arg Gly Ala Gly
 65 70 75 80

Ser Ser Gln His Gln Glu Arg Cys Cys Asn Glu Leu Asn Glu Phe Glu
 85 90 95

Asn Asn Gln Arg Cys Met Cys Glu Ala Leu Gln Gln Ile Met Glu Asn
 100 105 110

Gln Ser Asp Arg Leu Gln Gly Arg Gln Gln Glu Gln Gln Phe Lys Arg
 115 120 125

Glu Leu Arg Asn Leu Pro Gln Gln Cys Gly Leu Arg Ala Pro Gln Arg
 130 135 140

Cys Asp Leu Asp Val Glu Ser Gly Gly Arg Asp Arg Tyr
 145 150 155

<210> 5

<211> 1524

<212> DNA

<213> Peanut

<400> 5

cggcagcaac cggaggagaa cgcgtgccag ttccagcgcc tcaatgcgca gagacctgac 60
 aatcgcattg aatcagaggg cggttacatt gagacttga accccaacaa ccaggagttc 120
 gaatgcgccg gcgtcgccct ctctcgctta gtctccgcc gcaacgccct tcgtaggcct 180
 ttctactcca atgctcccca ggagatcttc atccagcaag gaaggggata ctttgggttg 240
 atattccctg gttgtcctag acactatgaa gagcctcaca cacaaggctc tcgatctcag 300
 tcccaaagac caccaagacg tctccaagga gaagaccaa gccaacagca acgagatagt 360
 caccagaagg tgcaccgttt cgatgagggt gatctcattg cagttccac cggtgttgct 420
 ttctggctct acaacgacca cgacactgat gttgttgctg tttctcttac tgacaccaac 480
 aacaacgaca accagcttga tcagttcccc aggagattca atttggctgg gaacacggag 540
 caagagttct taaggtacca gcaacaaagc agacaaagca gacgaagaag cttaccatat 600
 agcccatata gcccgcaaag tcagcctaga caagaagagc gtgaatttag ccctcgagga 660
 cagcacagcc gcagagaacg agcaggacaa gaagaagaaa acgaagggtg aaacatcttc 720
 agcggcttca cgcggaggtt cctggaacaa gccttcagg ttgacgacag acagatagtg 780
 caaaacctaa gaggcgagac cgagagtga gaagaggag ccattgtgac agtgagggga 840
 ggctcagaa tcttgagccc agatagaaag agacgtgccg acgaagaaga ggaatacgat 900
 gaagatgaat atgaatacga tgaagaggat agaaggcgtg gcaggggaag cagaggcagg 960
 gggaatggta ttgaagagac gatctgcacc gcaagtgcta aaaagaacat tggtagaaac 1020
 agatcccctg acatctacaa ccctcaagct gggtcactca aaactgccaa cgatctcaac 1080
 cttctaatac ttaggtggct tggacctagt gctgaatatg gaaatctcta caggaatgca 1140
 ttgtttgtcg ctactacaa caccaacgca cacagcatca tatatcgatt gaggggacgg 1200
 gctcacgtgc aagtcgtgga cagcaacggc aacagagtgt acgacgagga gcttcaagag 1260
 ggtcacgtgc ttgtggtgcc acagaacttc gccgtcgctg gaaagtccca gagcgagaac 1320
 ttgaatacgt tggcattcaa gacagactca agggccagca tagccaacct cgccggtgaa 1380
 aactccgtca tagataacct gccggaggag gtggttgcaa attcatatgg cctccaaagg 1440
 gagcaggcaa ggcagcttaa gaacaacaac cccttcaagt tcttcgttcc accgtctcag 1500
 cagtctccga gggctgtggc ttaa 1524

<210> 6

<211> 510

<212> PRT

<213> Peanut

<220>

<221> PEPTIDE

<222> (33) .. (47)

<223> peptide 1

<220>

<221> PEPTIDE

<222> (240) .. (254)

<223> peptide 2

<220>

<221> PEPTIDE

<222> (279) .. (293)

<223> peptide 3

<220>

<221> PEPTIDE

<222> (303) .. (317)

<223> peptide 4

<400> 6

Ile Ser Phe Arg Gln Gln Pro Glu Glu Asn Ala Cys Gln Phe Gln Arg

1

5

10

15

Leu Asn Ala Gln Arg Pro Asp Asn Arg Ile Glu Ser Glu Gly Gly Tyr

20

25

30

Ile Glu Thr Trp Asn Pro Asn Asn Gln Glu Phe Glu Cys Ala Gly Val
 35 40 45

Ala Leu Ser Arg Leu Val Leu Arg Arg Asn Ala Leu Arg Arg Pro Phe
 50 55 60

Tyr Ser Asn Ala Pro Gln Glu Ile Phe Ile Gln Gln Gly Arg Gly Tyr
 65 70 75 80

Phe Gly Leu Ile Phe Pro Gly Cys Pro Arg His Tyr Glu Glu Pro His
 85 90 95

Thr Gln Gly Arg Arg Ser Gln Ser Gln Arg Pro Pro Arg Arg Leu Gln
 100 105 110

Gly Glu Asp Gln Ser Gln Gln Gln Arg Asp Ser His Gln Lys Val His
 115 120 125

Arg Phe Asp Glu Gly Asp Leu Ile Ala Val Pro Thr Gly Val Ala Phe
 130 135 140

Trp Leu Tyr Asn Asp His Asp Thr Asp Val Val Ala Val Ser Leu Thr
 145 150 155 160

Asp Thr Asn Asn Asn Asp Asn Gln Leu Asp Gln Phe Pro Arg Arg Phe
 165 170 175

Asn Leu Ala Gly Asn Thr Glu Gln Glu Phe Leu Arg Tyr Gln Gln Gln
 180 185 190

Ser Arg Gln Ser Arg Arg Arg Ser Leu Pro Tyr Ser Pro Tyr Ser Pro
 195 200 205

Gln Ser Gln Pro Arg Gln Glu Glu Arg Glu Phe Ser Pro Arg Gly Gln
 210 215 220

His Ser Arg Arg Glu Arg Ala Gly Gln Glu Glu Glu Asn Glu Gly Gly
 225 230 235 240

Asn Ile Phe Ser Gly Phe Thr Pro Glu Phe Leu Glu Gln Ala Phe Gln
 245 250 255

Val Asp Asp Arg Gln Ile Val Gln Asn Leu Arg Gly Glu Thr Glu Ser
 260 265 270

Glu Glu Glu Gly Ala Ile Val Thr Val Arg Gly Gly Leu Arg Ile Leu
 275 280 285

Ser Pro Asp Arg Lys Arg Arg Ala Asp Glu Glu Glu Glu Tyr Asp Glu
 290 295 300

Asp Glu Tyr Glu Tyr Asp Glu Glu Asp Arg Arg Arg Gly Arg Gly Ser
 305 310 315 320

Arg Gly Arg Gly Asn Gly Ile Glu Glu Thr Ile Cys Thr Ala Ser Ala
 325 330 335

Lys Lys Asn Ile Gly Arg Asn Arg Ser Pro Asp Ile Tyr Asn Pro Gln
 340 345 350

Ala Gly Ser Leu Lys Thr Ala Asn Asp Leu Asn Leu Leu Ile Leu Arg

355

360

365

Trp Leu Gly Leu Ser Ala Glu Tyr Gly Asn Leu Tyr Arg Asn Ala Leu

370

375

380

Phe Val Ala His Tyr Asn Thr Asn Ala His Ser Ile Ile Tyr Arg Leu

385

390

395

400

Arg Gly Arg Ala His Val Gln Val Val Asp Ser Asn Gly Asn Arg Val

405

410

415

Tyr Asp Glu Glu Leu Gln Glu Gly His Val Leu Val Val Pro Gln Asn

420

425

430

Phe Ala Val Ala Gly Lys Ser Gln Ser Glu Asn Phe Glu Tyr Val Ala

435

440

445

Phe Lys Thr Asp Ser Arg Pro Ser Ile Ala Asn Leu Ala Gly Glu Asn

450

455

460

Ser Val Ile Asp Asn Leu Pro Glu Glu Val Val Ala Asn Ser Tyr Gly

465

470

475

480

Leu Gln Arg Glu Gln Ala Arg Gln Leu Lys Asn Asn Asn Pro Phe Lys

485

490

495

Phe Phe Val Pro Pro Ser Gln Gln Ser Pro Arg Ala Val Ala

500

505

510

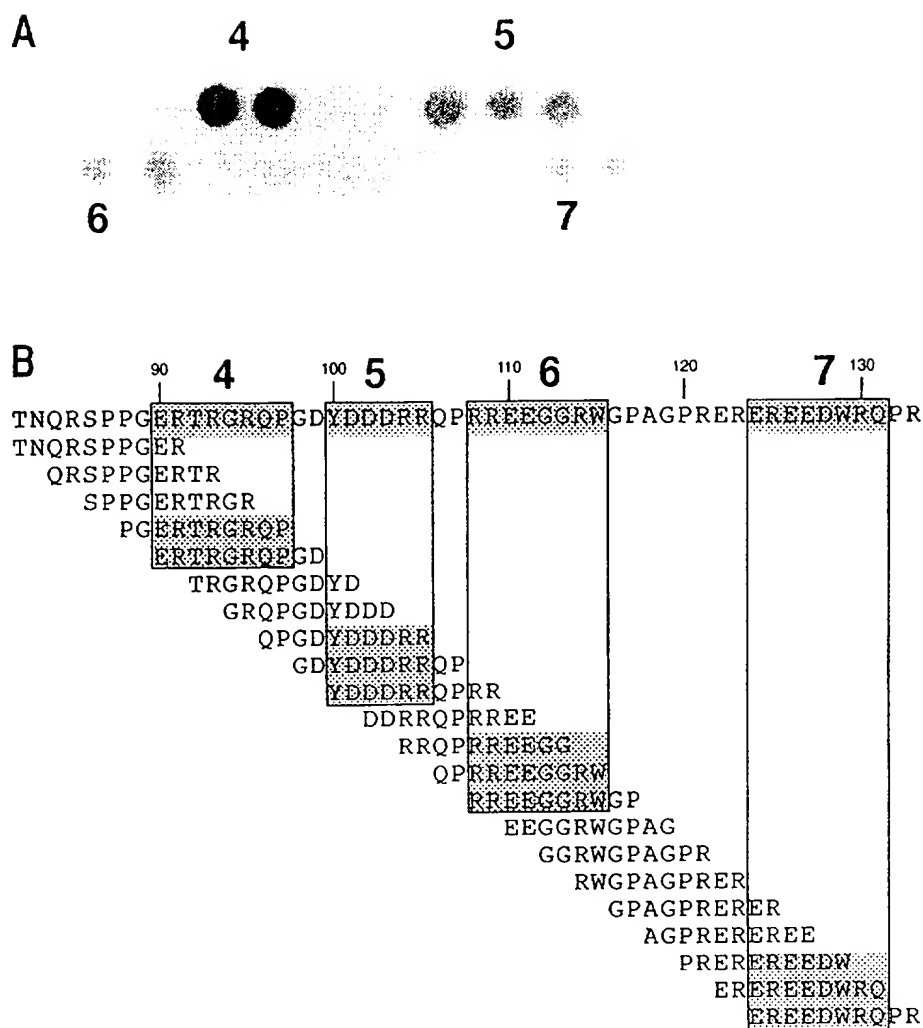


Fig. 1. Core IgE-binding epitopes identified on the Ara h 1 allergen. Epitopes analysis was performed on the IgE-binding sites by synthesizing 10 amino acid long peptides offset by two amino acids. These peptides were then probed with the 15 patient serum pool. (A) The peptide analysis of Ara h 1 amino acid residues 82-133. This region contains peptides 4, 5, 6 and 7 identified in Table 1. (B) The amino acid sequence of this region.

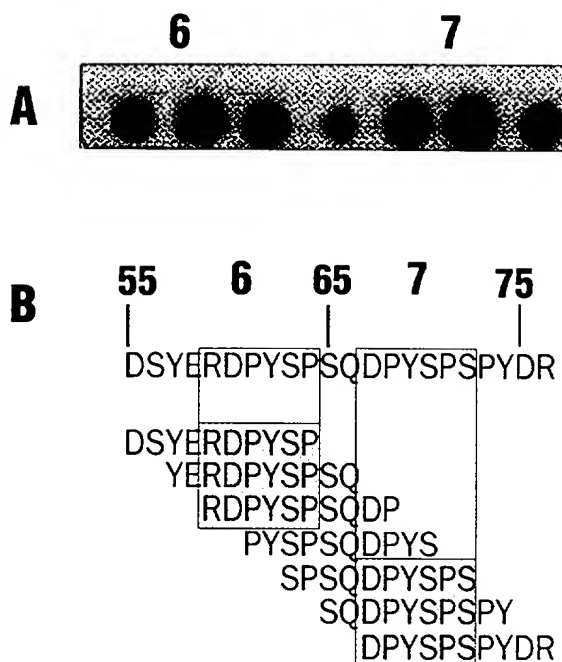


Fig. 2. Core IgE-binding epitopes identified on the Ara h 2 allergen. Epitopes analysis was performed on the IgE-binding sites by synthesizing 10 amino acid long peptides offset by two amino acids. These peptides were then probed with the 15 patient serum pool. (A) The peptide analysis of Ara h 2 amino acid residues 55-76. This region contains peptides 6 and 7 identified in Table 2. (B) The amino acid sequence of this region.

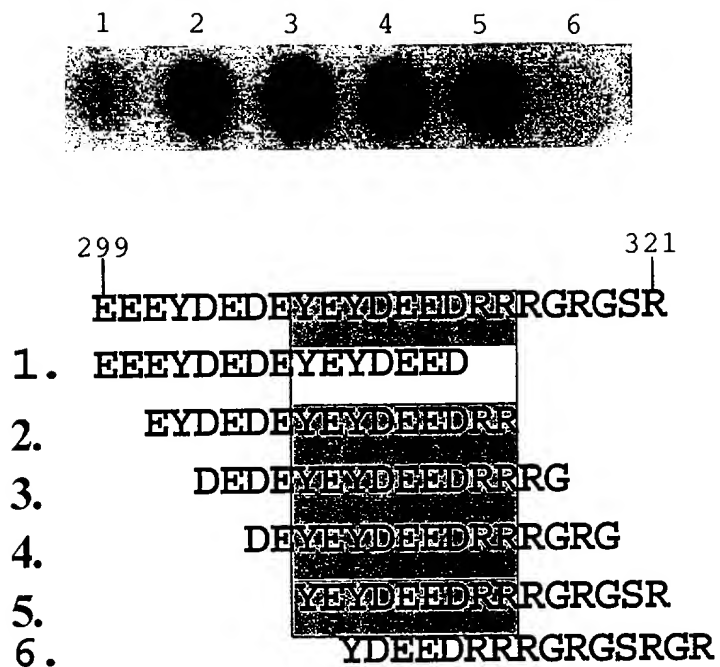


Fig. 3. Core IgE-binding epitopes identified on the Ara h 3 allergen. Epitopes analysis was performed on the IgE-binding sites by synthesizing 15 amino acid long peptides offset by two amino acids. These peptides were then probed with the 15 patient serum pool. (A) The peptide analysis of Ara h 3 amino acid residues 299-321. This region contains peptides 4 identified in Table 3. (B) The amino acid sequence of this region.

Epitope 9

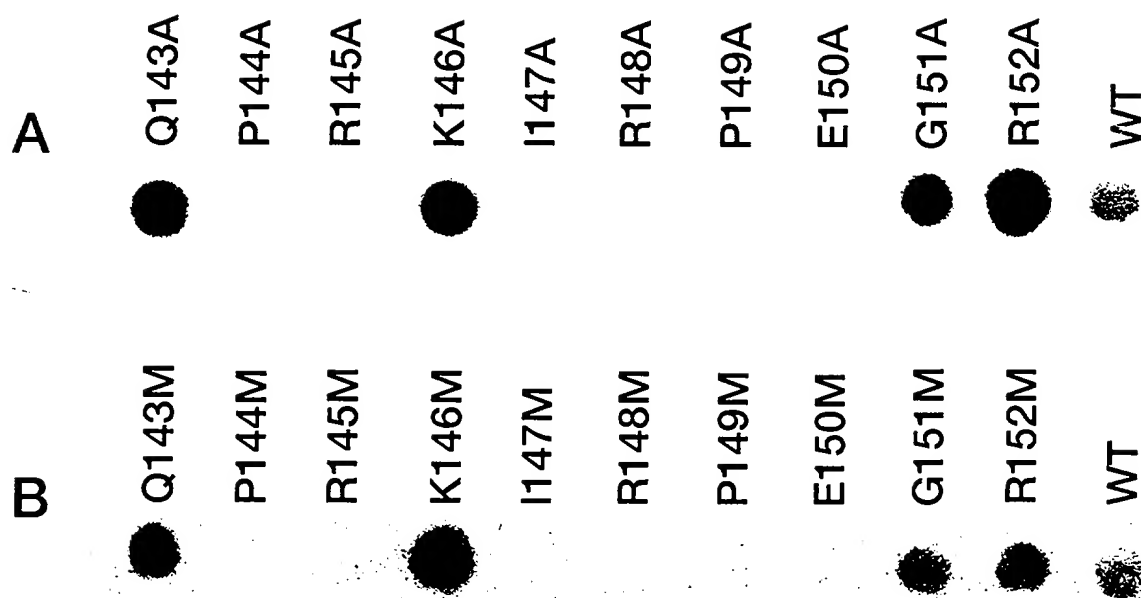


Fig. 4. Single amino acid changes to Ara h 1 epitope 9 result in loss of IgE binding to this epitope. Epitope 9 was synthesized with an alanine (Panel A) or methionine (Panel B) residue substituted for one of the amino acids and probed with a pool of serum IgE from 15 patients with documented peanut hypersensitivity. The letters across the top of each panel indicate the one letter amino acid code for the residue normally at the position and the amino acid that was substituted for this residue. The numbers indicate the position of each residue in the Ara h 1 protein. WT, indicates the wild type peptide (no amino acid substitutions).

Fig. 5. A modified Ara h 2 protein binds less IgE than the wild type Ara h 2 allergen. The Ara h 2 amino acid sequence is represented as the single letter amino acid code. Alanine was substituted for the wild type residue at the position indicated (Upper Panel). Relative amounts of IgE binding to modified Ara h 2 proteins were quantitated and expressed as a percentage of binding to the wild type allergen (Middle Panel).. Immunoblot analysis of IgG binding to wild tpe and modified Ara h 2 proteins showing that this immunoglobulin still binds the modified protein (Lower Panel).

Ara h 2 Amino Acid Sequence

```

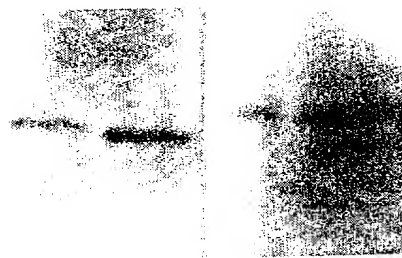
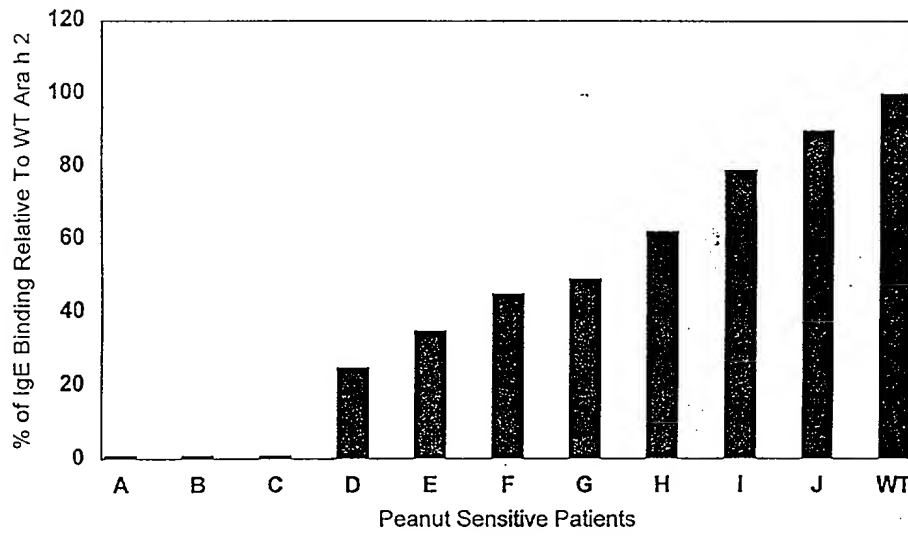
      A      A
      |      |
LTILVALALFLLAHASARQQWELQGDRRCQSQLERA-37
      A      A
      |      |
NLRPCEQHLMQKIQRDEDSYERDPYSPSQDPYSPSPY-74

DRRGAGSSQHQRCCNELNEFENNQRCMCEALQQIME-111

NQSDRLQGRQQEQQFKRELRLNLPQQCGLRAPQRCDL-148

VESGGRDRY-157
  
```

IgE Binding To Mutant Ara h 2



WT MUT WT MUT

Individual

Pool

T-Cell Proliferation Assay

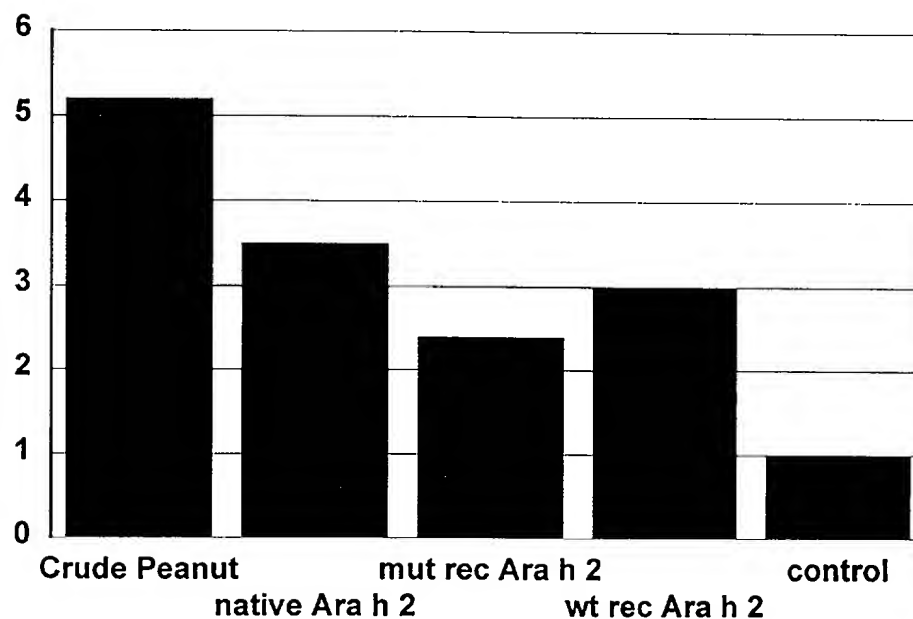


Fig. 6. The peripheral blood lymphocytes (PBLs) of peanut sensitive patients were isolated from whole blood using standard procedures of ficoll hypaque underlay. Cells were washed and suspended in media at a concentration of 4×10^6 cell/ml. One ml aliquots were placed in 24 well tissue culture plates and stimulated with 50 mg/ml of crude peanut extract (CPE) in order to establish peanut-specific cell lines. For the proliferation assays, T-cell lines at 2×10^5 cells/well were stimulated with either media alone (control), CPE (50 μ g/ml), native Ara h 2 (10 μ g/ml), mutant recombinant Ara h 2 (10 μ g/ml), or wild type recombinant Ara h 2 (10 μ g/ml) and allowed to proliferate for 6 days. On the last day the cells were incubated with [3 H]-thymidine (1 mCi/well) for 6-8 hours and then harvested onto glass fiber filters (Packard, Meriden, CT). T-cell proliferation was estimated by quantitating the amount of [3 H]-thymidine incorporation into cellular DNA. [3 H]-thymidine incorporation is expressed as a stimulation index which is defined as the fold-increase above media-treated cells.

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below), or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHODS AND REAGENTS FOR DECREASING CLINICAL REACTION TO ALLERGY

the specification of which (check one)

_____ is attached hereto

X was filed on August 27, 1998
as application Serial No. EM500208741US

_____ and was amended on: _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

| Prior Foreign Application(s) | | | Priority Claimed |
|------------------------------|--------------------|---------------------------------|---------------------|
| _____ (Number) | _____ (Country) | _____ (Day/Month/Year Filed) | _____ |
| _____ (Number) | _____ (Country) | _____ (Day/Month/Year Filed) | _____ |
| _____ (Number) | _____ (Country) | _____ (Day/Month/Year Filed) | _____ |

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

| Provisional Application Number | Filing Date |
|--------------------------------|--|
| <u>60/074,590</u> (Number) | <u>February 13, 1998</u> (Day/Month/Year Filed) |
| <u>60/074,624</u> (Number) | <u>February 13, 1998</u> (Day/Month/Year Filed) |
| <u>60/074,633</u> (Number) | <u>February 13, 1998</u> (Day/Month/Year Filed) |

Se Di

Title "METHODS AND REAGENTS FOR DECREASING CLINICAL REACTION TO ALLERGY"
By Gary A. Bannon, A. Wesley Burks, Hugh Sampson, Howard Sosin
Filed August 27, 1998
DECLARATION

Full name of second inventor A. Wesley Burks

Inventor's signature _____ Date _____

Residence 2400 North Pierce, Little Rock, Arkansas 72207

Citizenship U.S.

Post Office Address Same

Full name of third inventor Hugh Sampson

Inventor's signature _____ Date _____

Residence 19 Carleon Avenue, Larchmont, New York 10538

Citizenship U.S.

Post Office Address Same

Full name of fourth inventor Howard Sosin

Inventor's signature _____ Date _____

Residence 640 Sasco Hill Road, Fairfield, Connecticut 06430

Citizenship U.S.

Post Office Address Same